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13443

Capillary Tube Kjeldahl Method for Determining Protein Content of 5 to 20 Milligrams of Tissue Fluid.

OTTO SCHALES, RICHARD V. EBERT AND EUGENE A. STEAD, JR.
(Introduced by Soma Weiss.)

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A study of the protein content of tissue and edema fluid has been hampered in the past because of the difficulty in obtaining large enough quantities of fluids for satisfactory analysis. In many instances it is easy to obtain a few milligrams of clear fluid, but attempts to collect large amounts usually lead to bleeding. The pur-

pose of this report is to present a technic for collecting small amounts of fluid from the tissues and to describe a method for determining the nitrogen content of this fluid.

To collect the fluid, several 23-gauge 5/8-inch needles are inserted to the hilt into the subcutaneous tissue. If the part is not dependent at the time the needles are inserted, contamination with blood usually does not occur. The needles are then withdrawn about 5 mm and allowed to remain in place for 5 to 10 minutes, unless fluid appears in the hub of the needle before that time. If the needles are left in place for a long time, an inflammatory reaction may result and a fluid high in protein will be obtained. The needles are then withdrawn and attached to a syringe. Gentle pressure on the plunger of the syringe forces the fluid to the tip of the needle. From there the drop of fluid obtained is transferred by capillary attraction to a clean glass capillary tube of known weight. The fluid should be clear. If there is any question of contamination with blood, the fluid can be examined for red cells by placing the capillary tube under the microscope. Red cells are easily visible through the walls of the glass tube. After the weight of the filled capillary tube has been determined, the material is blown into a small pyrex test tube which contains 0.1 ml sulphuric acid (concentrated sulphuric acid diluted 1:1 with water). The content of the capillary tube is fairly quantitatively washed out by drawing the acid up and down several times. The capillary tube is then washed, dried and weighed. The mixture in the test tube is concentrated over a microburner until dense white fumes appear. Digestion is continued for 10 minutes. The flame is then removed and about ½ minute later, .02 cc of 30% nitrogen-free hydrogen peroxide is added. The heating is now continued for 5 minutes in order to complete the digestion. After cooling the tube, 4 cc of distilled water and 0.1 cc of gum ghatti solution are added. A color equivalent to the amount of nitrogen present is produced by adding 1.6 ml of Nessler's reagent (prepared according to Koch and McMeekin[1]). After mixing, the contents of the digestion tube are transferred to a Klett colorimeter tube and read with filter 42 (transmission limits 400 to 450 millimicrons). A blank is prepared in exactly the same way, except for the addition of the edema fluid.

The amount of nitrogen per 100 g is calculated as follows:

$$\frac{(\text{Reading of unknown} - \text{Reading of blank}) \times \text{calibration factor} \times 100}{\text{Weight of sample in milligrams}}$$

= mg nitrogen in 100 g of sample. It is not necessary to determine the amount of non-protein nitrogen in the fluid because the non-

[1] Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066.

TABLE I.
Determination of Calibration Factor.
Ammonium sulphate solution 0.6630 g in 100 cc of water = 0.01405 g of nitrogen in 100 cc of water.

Weight of sample (mg)	Nitrogen in sample (μg)	Klett reading (reading of blank subtracted)	Calibration factor
9.0	12.7	250	.0508
6.9	9.7	181	.0536
7.6	10.7	200	.0535
8.3	11.7	230	.0509
9.9	13.9	260	.0535
		Avg	.0525

protein nitrogen of edema fluid and of plasma are practically the same.[2] The non-protein nitrogen of the serum or plasma of the patient from whom the fluid was obtained is then determined and this is subtracted from the milligrams of nitrogen in 100 cc of the sample. The concentration of protein in grams percent in the fluid is then calculated in the usual way. The calibration factor is determined by running ammonium sulphate solutions of known concentration through the entire procedure, including the transfer of a drop of such a solution from a capillary tube to a digestion tube (Table I).

The protein content of 5 different sera diluted 1:6 with saline was determined by a standard micro-Kjeldahl procedure,[1] with the Klett

TABLE II.
Protein Concentration of 5 Sera (Diluted 1:6) as Determined by Capillary Method and by Micro-Kjeldahl Method.

Sera diluted 1:6	Capillary Kjeldahl		Micro-Kjeldahl Protein (g%)
	Mg of fluid	Protein (g%)	
1	9.5	1.0	1.0
	9.0	1.0	
	9.1	1.0	
2	11.7	1.0	1.1
	8.7	1.0	
	11.2	1.0	
3	10.8	1.0	1.1
	10.5	1.0	
	7.6	0.9	
4	7.5	1.1	1.2
	10.2	1.1	
	8.7	1.2	
5	11.2	0.9	1.0
	11.6	1.0	
	11.5	0.9	

2 Denis, W., and Minot, A. S., *Arch. Int. Med.*, 1917, **20**, 879.

colorimeter and filter 42, and by the capillary tube technic (Table II). The micro-Kjeldahls were done in duplicate and checked within 2%. The capillary tube Kjeldahls were done in triplicate, as shown in Table II. The results were either the same or slightly lower than those obtained by the micro-Kjeldahl method. The greatest difference was .2 g, and this occurred in only 1 determination out of 15. If sufficient fluid is obtained to make the determination in duplicate, it seems logical to take the higher of the 2 readings as the more nearly correct one.

Clear edema fluid can usually be obtained without difficulty if there is sufficient edema to cause even slight pitting. Table III gives protein content of the edema fluid of 6 patients.

Summary. A technic is given for obtaining 5 to 20 mg of subcutaneous fluid in subjects with minimal edema. A capillary tube Kjeldahl method for determining the protein content of small amounts of edema fluid is described.

This work was done with the technical assistance of Rosamond Piotti, S.B.

TABLE III.
The Protein Content of Edema Fluid of 6 Patients.

Subject	Diagnosis	Mg of edema fluid	Klett reading (reading for blank subtracted)	Protein (g%)
St	Chronic congestive failure	11.3	636	1.8
		9.0	586	1.8
W	Chronic congestive failure	10.4	128	.2
		13.4	185	.3
B	Cirrhosis of liver	12.4	141	.2
		8.7	95	.2
Sh	Myxedema and vitamin deficiency	7.5	328	1.3
		6.4	288	1.4
O	Emphysema	13.7	104	.1
		6.2	50	.1
C	Serum sickness	2.6	383	5.1

13444

Action of Drugs Containing Basic Nitrogen on d-Amino Acid Dehydrogenase.

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A complete analysis of the actions of a drug has come to include information regarding the effect of the drug upon the metabolism of surviving tissue slices and upon the catalytic properties of isolated enzyme systems. In this paper the effects of certain drugs upon the conversion of d-alanine to pyruvic acid with concomitant utilization of oxygen in the presence of the enzyme d-amino acid dehydrogenase are reported. The drugs studied contained basic nitrogen and were chosen to include different types of functional nitrogen. Krebs[1] has already reported the inability of veronal to modify the catalysis of deamination of d-amino acids by d-amino acid dehydrogenase in experiments in which the barbiturate was used in a buffer system. Klein and Kamin[2] reported the failure of a large group of compounds containing nitrogen to modify the action of d-amino acid dehydrogenase.

Method. The enzyme was prepared by the method of Krebs.[1] The minced hog kidneys came in contact with acetone within 15 minutes after the death of the animals. The extracts were prepared by shaking 1 g of the dried powdered kidneys in 40 ml of distilled water for 10 minutes. After centrifugation for 10 minutes the supernatant extract was pipetted off. It contained 4.04 mg protein per ml and was as active as similar extracts reported by other workers.

The utilization of oxygen in the deamination of d-alanine in the presence of the extract was measured by the method of Warburg,[3] in the presence of oxygen at 37°C (±.02°). The extract was buffered with Na_2HPO_4 in the reaction vessels. After attainment of equilibrium, substrate, also buffered with Na_2HPO_4, was added to the control vessel from the side arm. The final concentration of dl-alanine was 4.4×10^{-2} M; of phosphate, 0.034 M. In the case of the experimental vessels similarly buffered solutions containing both dissolved substrate and drug were introduced from the side arms.

1 Krebs, H. A., *Biochem. J.*, 1935, **29**, 1620.

2 Klein, J. R., and Kamin, H., *J. Biol. Chem.*, 1941, **138**, 507.

3 Warburg, O., *Über den Stoffwechsel der Tumoren*, Berlin, 1926.

Final experimental concentrations of substrate were 4.4×10^{-2} M; of phosphate, 0.034 M; and of drugs, 2×10^{-3} M. Such concentrations of drug have been found acceptable in our[4] studies of the action of drugs on surviving tissue slices and are in the same range as those of certain therapeutic agents in the blood-stream during medication. Furthermore such concentrations do not, at the pH finally attained, introduce too great danger of precipitation either of drug or of dispersed protein. The values of final pH for each system will be found in the tabulated results.

Our experience with presumably pure substrate disclosed that the quantitative and even the qualitative nature of the action of quinidine on the dehydrogenase depended upon the source of the sample of d-alanine. For example, with one substrate sample, quinidine augmented the catalytic action of d-amino acid dehydrogenase while, with another, it depressed it. These observations could be reproduced at will under rigorously controlled conditions in which checks did not vary by more than 2%. To arrive at results which represented the true action of the drug it was necessary to use samples of d-alanine purified by 4 recrystallizations from aqueous alcohol. Continued purification of each of 3 samples of dl-alanine led to a decrease in and finally to a disappearance of discrepancy in behavior. That is, after final recrystallization each sample of alanine yielded a reaction which could be qualitatively and quantitatively reproduced with the other samples. The commercial samples were procured from Eastman Kodak Co. and from Hoffman-LaRoche Co. The other sample was prepared in this laboratory by the acetaldehyde-ammonium cyanide reaction. The last preparation recrystallized 4 times after its original presumable purification served as the substrate on which we report. It is important to note that results obtained from an improperly purified substrate might be ascribed by the observer to properties of the enzyme rather than to those of the substrate except in those cases where the action of a drug chanced to disclose the real nature of the situation. We are not yet prepared to state why substrates from different sources produce such variations in results.

Since it is not possible to secure final conditions in which concentrations of all electrolytes, concentrations of substrate, and pH are identical, it was elected to gain the former identities and to make interpretation in the light of slight differences in final pH. For example, with the system containing quinidine sulfate the average

[4] Rice, J. C., and Brazda, F. G., *Proc. Am. Physiol. Soc.*, p. 234, Chicago, Ill., April, 1941.

of the initial and final pH values was 7.98 (glass electrode), while that of the control was 8.04. In spite of the fact that the latter pH is more nearly optimal the systems containing quinidine led to a greater utilization of oxygen by 9%. This augmentation is easily reproducible; it represents the limiting value of the activity of systems in which substrates from different sources and in different states of purity are used; it transcends the variation which arises from the method as we use it by 7%. It must unquestionably represent a property of quinidine sulfate as related to the action of d-amino acid dehydrogenase. Its significance rests upon the results from at least 20 determinations.

Results. Calculated in terms of gross oxygen utilization over a one-hour period in control situations our extracts demanded 500-600 cmm of O_2 per 2 ml aliquot on different occasions. This range of oxygen utilization arises from difference in fineness of powdering, unavoidable variable hysteresis of each dispersed extract, and the effect of time in causing deterioration of the powdered kidney. The variability is not related to the interpretation of experimental results because the action of a drug was compared with controls which utilized the identical extract. The results are expressed as percentage augmentation or depression and are found in Table I. Determinations for each drug were reproduced at least 4 times except those for quinidine which demanded the more extensive treatment already described above.

The tabulated results indicate an augmentation by veratrine amounting to 6%. This exceeds by 4% the variation which we are accustomed to expect with this method. Bernheim and Bernheim[5] have reported that veratrine inhibits the oxidation of proline by

TABLE I.

Drug	Effective concentration	Action on catalysis by d-amino acid dehydrogenase	Avg of Initial and final pH
Control	—	—	8.04
Quinidine (as sulfate)	2×10^{-3} M	9% augmentation	7.98
Veratrine (as sulfate)	,, ,,	6% ,,	7.94
Ephedrine (as sulfate)	,, ,,	3% ,,	7.84
Sulfanilamide	,, ,,	3% ,,	7.84
Pilocarpine (as hydrochloride)	,, ,,	3% inhibition	7.65
Morphine (as sulfate)	,, ,,	No change	7.81
Physostigmine (as sulfate)	,, ,,	,, ,,	7.75
Quinine (as sulfate)	,, ,,	,, ,,	7.98
Riboflavin	Kidney extract saturated with riboflavin	,, ,,	8.01

5 Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Therap.*, 1933, **48**, 73.

liver tissue. It is possible that with higher concentrations of veratrine this might have happened to the action of d-amino acid dehydrogenase on d-alanine. With such concentrations there might have arisen, however, the hazard of incipient precipitation of either protein or drug, a hazard which we strove to avoid.

Quinidine augmented the reaction more strongly than veratrine. Quinine, however, failed to cause any measurable modification of the utilization of oxygen. It must be pointed out, however, that since incipient precipitation of quinine resulted in every experiment the critical concentration may not have been reached. Since the utilization of oxygen in the system containing quinine was not different from that in the control vessels, the material precipitated could hardly have been enzyme-bearing proteins. It was observed repeatedly in experiments employing pH values around 8.0 and a milieu of PO_4 ion that it is not possible to exceed the concentrations of the sulfate of quinine and quinidine which we employed. The stereoisomeric difference between quinine and quinidine is sufficiently fundamental to produce the difference observed.

Conclusions. If one excludes the effects on pH which are produced in the reacting systems by the presence of drugs, the results indicate that ephedrine, morphine, physostigmine, pilocarpine, riboflavin, and sulfanilamide do not modify the catalysis of the dehydrogenation of d-alanine by d-amino acid dehydrogenase. The augmentations produced by quinidine and veratrine, although small, are significant.

13445 P

A New Growth Factor for Hemolytic Streptococci.

N. GROSSOWICZ. (Introduced by I. J. Kligler.)

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Reports have appeared on cultivation of *Str. hemolyticus* in synthetic or semisynthetic media to which growth accessory factors were added. Woolley and Hutchings[1] grew strains of Lancefield's B and D types on a medium consisting of amino-acids, glucose, salts and riboflavin, pantothenic acid, pyridoxin and reduced iron; groups

[1] Woolley, D. W., and Hutchings, Brian, L., *J. Bact.*, 1940, **39**, 287.

A, C and E failed to grow on this medium. McIlvain[2] confirmed these results with the addition of glutamin. Pappenheimer and Hottle[3] grew type A hemolytic streptococcus in a medium which contained, in addition to accessory substances used by Woolley, nicotinic acid, thiamin, biotin and some purines. Woolley[4] reported on the isolation from liver of a new growth factor for *Str. hemolyticus* type A. Type A streptococcus failed to grow in the medium of Pappenheimer and Hottle[3] unless this factor was added.

This report describes the properties of what appears to be a new factor, which gave good growth of *Str. hemolyticus,* type A.

1. The basic medium consisted of:

Acid hydrolized casein	3.0 g
Glucose	1.0 "
NaCl	5.0 "
$Na_2HPO_4 \cdot 12\ H_2O$	2.5 "
KH_2PO_4	0.35 "
$MgCl_2 \cdot 6\ H_2O$	0.3 "
Fe and Mn	trace
H_2O (distilled)	1000 cc

2. The solution (pH 7.4) was sterilized by filtration. The strains were freshly isolated from patients and gave typical β-hemolysis. The strain most used was H2682, kept in broth + 10% serum.

3. The growth factor, derived from tomato juice passed through a meat grinder and filtered. The juice is acid and sterilized by filtering through Seitz filter or triple sterilization in Arnold sterilizer. Addition of 0.05 cc of the neutralized juice to 10 cc of basic medium gives visible growth, while 0.3 cc gives abundant growth. Addition of nicotinic acid, thiamin, riboflavin, ascorbic acid, β-alanin, thioglycolic acid, p-aminobenzoic acid, glutamine, adenine and urazil singly and in combination failed to give visible growth.

The growth substance was purified by the following procedure: (a) Lead acetate in neutral or alkaline reactions precipitates only inactive substances, the active factor remaining in solution. (b) Alcohol (6 vol. to 1) also precipitates the inactive substances. (c) Aceton, 1:1, precipitates only inactive substances; in large excess it precipitates all the active substance. (d) Norit adsorbs the active substance quickly and completely at various reactions (pH 3.0, 7.0, 9.0); Fuller's earth, Kaolin, Kieselgur and talcum fail to adsorb it; animal charcoal adsorbs it only partially and irregularly. (e) Elution from the Norit is affected by the addition of the original volume of

2 McIlvain, H., *Brit. J. Exp. Path.*, 1940, **21**, 25.

3 Pappenheimer, A. M., Jr., and Hottle, G. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 645.

4 Woolley, D. W., *J. Exp. Med.*, 1941, **73**, 487.

80% alcohol containing 1.5% ammonia; about 50% of the active substance can thus be eluted.

These properties were utilized in the purification of the growth factor from the crude tomato juice by the following steps:

1. One l juice concentrated *in vacuo* to 200 cc.
2. One l 96% alcohol added, left over night.
3. The clear decanted supernatant concentrated *in vacuo* to 200 cc.
4. Two l acetone added and left over night.
5. Active precipitate dissolved in 1 l distilled water containing 70 cc H_2SO_4 (pH 2.0).
6. 25 g Fuller's earth added, shaken 30 minutes and filtered.
7. Added 20 g Norit to active acid filtrate, mixture shaken 1 hr and filtered. Filtrate inactive.
8. Norit adsorbate extracted with 1 l alcohol-ammonia sol. during 3-4 hr and filtered.
9. Alkaline filtrate active; immediately concentrated in vacuum.

The neutralized active eluate added to the basic medium without any other growth factor gives visible growth. Addition of riboflavin intensifies the growth, but the addition of the various other growth factors mentioned above had no such effect.

The final eluate had a deep yellow color. The addition of 0.6 cc of this eluate corresponding to 0.3 cc of the original crude tomato extract to 10 cc of the medium yielded good growth.

The purified active substance like that of the crude juice withstood boiling at pH 7.0 or 4.0, for at least 6 hours, but was destroyed in autoclave; at pH 4.0 at room temperature the substance retains its activity for a long time. At pH 9.0 it is much less resistant to boiling; at this reaction the potency is lost after 2 to 3 weeks at room temperature. Boiling half an hour in 0.5 N HCl or 0.5 N NaOH destroys the active substance.

Heating in 3% K MnO_4 or in 3% H_2O_2 destroys the active substance; Br_2 destroys it quickly at room temperature.

The active principle is sensitive to nitrous acid and formaldehyde even at room temperature; 0.1% formaldehyde caused prompt inactivation.

The reactions with nitrous acid and formaldehyde indicate that the active factor contains an NH radical.

The presence of an amino group suggests a relationship to biotin. However, this substance differs from biotin for it is less resistant to heating and particularly to alkaline reactions. The same difference exists in relation to the butyl factor of Woolley[5] and the B-Y factor

[5] Woolley, D. W., McDaniel, L. E., and Peterson, W. H., *J. Biol. Chem.*, 1939, **131**, 381.

of Oxford[6] for anaerobes. It is not identical with glutamine and p-amino benzoic acid because these substances have no activating effect on the growth of *Str. hemolyticus*.

13446

Comparative Potentiating Effects of Certain Therapeutic Agents on Sodium Evipal Hypnosis.

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Barlow and Gledhill[1] demonstrated the potentiation of isoamyl-ethyl barbituric acid and ethyl (1-methyl butyl) barbiturate on morphine. More recently, Loewe[2] utilized this principle to show that active cannabis preparations, when administered orally to mice, materially prolonged the hypnotic effect of subcutaneously administered pernoston.

We have observed that the intravenous administration of evipal sodium under carefully standardized conditions produces a remarkably consistent reaction on the adult male rat. This is particularly true if the *initial* voluntary movement is taken as the criterion of recovery from hypnosis. Female rats differ materially from males in their reactions to evipal in that hypnosis is more irregular and persists 2 to 5 times as long. Immature animals react inconsistently.

Materials and Methods. Adult male rats, weighing from 325-400 g were used throughout this study; all animals were fasted for 14-18 hours prior to medication. The normal period of hypnosis from a standard dose of evipal sodium administered by the saphenous vein as a 4.0% aqueous solution at an injection rate of 0.1 cc per 15 seconds was determined. This period was estimated within 10 seconds by counting time from the establishment of hypnosis (the completion of the intravenous injection) until the *first* voluntary movement occurred following the injection. A period of 5-7 days was allowed to elapse before the animals were again medicated. The compound to be tested in conjunction with the hypnotic was adminis-

[6] Oxford, A. E., Lampen, J. O., and Peterson, W. H., *Biochem. J.*, 1940, **34**, 1588.

[1] Barlow, O. W., and Gledhill, J. D., *J. Pharm. and Exp. Therap.*, 1933, **49**, 36.

[2] Loewe, S., *J. Am. Pharm. Assn.*, 1940, **29**, 572.

tered orally or subcutaneously to fasted animals 30 minutes prior to the injection of the hypnotic by vein. In other experiments, the oral medication preceded the intravenous administration of the hypnotic by 60 minutes. The animals were again allowed to rest for a period of 5-7 days, when the hypnotic effect of the intravenously administered standardized dose of evipal sodium was again determined. Under such conditions, a single animal could be used for not less than 5 or 6 separate tests.

The method described above is amenable to graphic recording. The procedure[3] necessitates the restraint of the rat on its back by means of specially adapted animal boards and the recording of muscular movements on a smoked paper by means of a recording lever attached by thread to a small serrafine clamp, which in turn is attached to the anterior abdominal wall. During the period in which the animal is under the influence of evipal sodium, only respiratory movements are recorded. The end point is defined as that moment when voluntary movements reappear.

Results. The results are summarized in Table I.

Na Bromide: No potentiation was observed in spite of the large dose administered. This was felt to be due to the fact that insuf-

TABLE I.
Potentiation of Hypnotic Effect of Na Evipal by Various Therapeutic Agents.

No. of animals	Dose of Evipal Na (intravenous) mg per kg	Additional medication: Drug	Route	Dose, mg/kg	Mean duration of anesthesia (min.)	P.E.$_M$*
183	40	—	—	—	11.3	± 0.26
30	40	Na bromide	p.o.	350	11.4	± 0.36
33	40	" "	p.o.	350†	15.9	± 0.56
27	40	Amidopyrine	p.o.	250	19.1	± 0.57
21	40	Acetylsalicylic acid	p.o.	175	14.2	± 0.55
19	40	Morphine SO_4	s.c.	1.0	17.6	± 0.49
24	40	" "	s.c.	2.0	18.6	± 0.52
21	40	" "	s.c.	4.0	29.1	± 0.72
17	40	" "	s.c.	6.0‡	30.4	± 0.94
48	40	" "	s.c.	42.0§	37.2	± 1.12
26	40	Demerol (D-140)	p.o.	50	22.4	± 0.72
18	40	" "	p.o.	20	18.3	± 0.45
52	60	—	—	—	30.4	± 0.69
29	60	Demerol (D-140)	p.o.	50	87.7	± 3.88
29	60	Amidopyrine	p.o.	250	84.6	± 2.39

$$*\text{P.E.}_M = \frac{\sigma \times 0.6745}{\sqrt{n-1}}$$

†1 daily, 5 days.
‡2 deaths.
§35 deaths.

[3] Barlow, O. W., *J. A. M. A.*, 1932, **99**, 986.

ficient time was allowed for saturation of the tissues with bromide. Accordingly, a second group of animals was employed, which received daily doses of sodium bromide (350 mg per kg per day, p.o.) for a period of 5 days before the second dose of evipal sodium was administered. These animals showed no gross depression or impairment of spontaneous movements during the course of bromide medication. However, the potentiating effect of such continued therapy on evipal hypnosis is clearly demonstrable.

Acetylsalicylic Acid: A slight degree of potentiation resulted from the administration of this dose. No significant depression was observed in these animals prior to the administration of the evipal sodium.

Amidopyrine: Marked potentiation of the hypnotic effect was observed. This dose did not produce any significant depression.

Morphine: The initial dose of morphine as 10% of the lethal dose, based on a lethal dose of 420 mg per kg as established by Hunt,[4] caused 35 deaths in a group of 48 animals. We subsequently observed that this figure for the generally accepted M.L.D. of morphine was not applicable to our rat colony, a point which is borne out by the fact that doses as small as 1.0 mg per kg produced a definite synergistic effect with evipal by vein. This synergism became more pronounced as the dose level increased.

Demerol (D-140): This is a new type of potent analgesic originally described by Eisleb and Schaumann[5] and more recently by ourselves.[6] It is 1-methyl 4-phenylpiperidine 4-carbonic acid ethyl ester. A detailed account of the pharmacological properties of this material is now in preparation.[7] Single doses of this order have a marked analgesic effect, but only a slight sedative action. We have been able to demonstrate this sedation objectively by Barlow's[3] tranquilizing procedure. Under the conditions of this test, this drug has a definite potentiating effect on the hypnotic action of sodium evipal, which is superior to that of amidopyrine and approaches that of morphine.

Summary. The hypnotic effect of sodium evipal, administered intravenously, may be markedly potentiated by acetylsalicylic acid, amidopyrine, demerol (D-140) and morphine. The synergistic effectiveness is in the order named.

4 Hunt, R., *U. S. Hygiene Lab. Bull.* 1910, p. 83.

5 Eisleb, O., and Schaumann, O., *Deutsch. med. Wochschr.*, 1939, **65**, 967.

6 Climenko, D. R., and Barlow, O. W., in preparation.

7 Barlow, O. W., and Climenko, D. R., in preparation.

13447

Chemiluminescence of Alcaptonuric Urines.*

Lynn D. F. Abbott, Jr. (Introduced by J. C. Forbes.)

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That light is given off during the oxidation of homogentisic acid in alcaptonuric urines has not been reported previously. This peculiarity of alcaptonuria may be of importance as a possible explanation of instances of luminous urine mentioned in the older literature.[1, 2, 3] The luminescence of two different alcaptonuric urines, as well as of the homogentisic acid and lead homogentisate isolated from these urines, is described in this paper. The urines were those of 2 Negro children reported in an earlier study.[4]

When alcaptonuric urine is made alkaline, homogentisic acid is rapidly oxidized by the air and forms a brownish-black substance. It is during this oxidation that light is emitted. The observations were made in a completely dark (photographic) room after allowing 15 minutes for dark adaptation. To 5 cc of urine, 1 cc of 10% sodium hydroxide is added. The test tube is then covered with the thumb and shaken vertically with vigor. Only a faint glow usually comes on shaking the first time. On the second shaking, however, a white glow appears a fraction of a second after the shaking is stopped. The glow is especially strong in the froth layer but permeates the whole solution. The light fades rather rapidly, but there is an after-glow for some seconds after shaking. The urine can then be shaken again and the glow will return, and fade. This can be repeated 4 or 5 times. Apparently as long as some homogentisic acid remains unoxidized, luminescence will reappear on shaking.

Both alcaptonuric urines exhibited this phenomenon, the one containing the higher concentration of homogentisic acid giving the more luminescence. Normal urines, either fresh or old, did not luminesce when treated similarly. The oxidation of homogentisic acid by air takes place rapidly only in alkaline solutions.

Similar results were obtained with normal urines to which either

* The author is indebted to Professor E. Newton Harvey of Princeton University for suggesting that observations for luminescence be made on alcaptonuric urines.

1 McDermott, F. A., *J. Am. Chem. Soc.*, 1913, **35**, 824.

2 Harvey, E. N., *The Nature of Animal Light*, J. B. Lippincott Co., 1920, p. 17.

3 Harvey, E. N., *Living Light*, Princeton University Press, 1940, p. 21.

4 Abbott, L. D., Jr., *Science*, 1941, **94**, 365.

homogentisic acid or lead homogentisate had been added. Water solutions of these compounds likewise luminesce. Since there is no froth layer when water solutions are shaken, the addition of several drops of a froth-producing substance (*e. g.*, 2% egg albumin) greatly facilitates observation of the phenomenon as the froth layer, where the greatest oxidation no doubt occurs, gives off the most brilliant light.

It is also of interest that hydroquinone, which likewise oxidizes and blackens when treated in a similar manner, emits no light when treated similarly and thus differs in behavior from the substituted hydroquinone, homogentisic acid (hydroquinone acetic acid).

Summary. Alcaptonuric urines, as well as solutions of homogentisic acid or lead homogentisate, give off light when made alkaline and shaken with air.

13448

Effect of Congo Red on Plasma Prothrombin.*

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Congo red has been widely used as a hemostatic agent, but its mode of action remains obscure.[1] Wedekind, Becker and Wienert[2] first observed that Congo red injected intravenously had a hemostatic effect. The coagulation time was shortened. Blood platelets were increased 2 or 3 times the initial number by the 5th or 6th day and returned to the initial figure in 3 weeks. It has been suggested[1] that "the hemostatic action of Congo red may be related to a limitation of the function of the spleen, with resultant elevation of platelets in the blood." However, there are apparently no experimental studies that support such an opinion concerning the effect of Congo red.

Because of the reported beneficial results in controlling hemorrhage with Congo red, its effect on the level of plasma prothrombin was studied and compared to that of 2-methyl-1,4-naphthoquinone.

* The investigation has been supported by grants from The Lady Tata Memorial Trust and by a Fund for the Study of Leukemia.

1 Arons, I., and Sokoloff, B., *Am. J. Roent. and Rad. Ther.*, 1939, **41**, 834.

2 Wedekind, T., Becker, J., and Wienert, B., *Munchen Med. Wchnschr.*, 1930, **77**, 2049.

In order to obtain diminished plasma prothrombin levels mice were fed a diet containing mineral oil. It has recently been recorded that prothrombin deficiency in rats can be produced by feeding an adequate diet containing 20% by weight of mineral oil.[3]

In each experiment, the mice used were of the same stock and age (within one week). The diet of the control mice consisted of a mash of bread and milk, with dog biscuits in most experiments. Other mice received the same with mineral oil (C.P.) constituting 5, 10, or 20% by volume of the mash.

Mice were killed by ether and approximately 1.2 cc of blood (usually from 2 mice) was obtained by cardiopuncture and added to 0.2 cc of 1.5% sodium oxalate solution. The plasma prothrombin level was determined by the method of Warner, Brinkhous and Smith.[4] We are indebted to Miss Florence Stevens and Miss Virginia Hewitt of the Department of Surgery of the New York Hospital and Cornell University Medical College for these determinations.

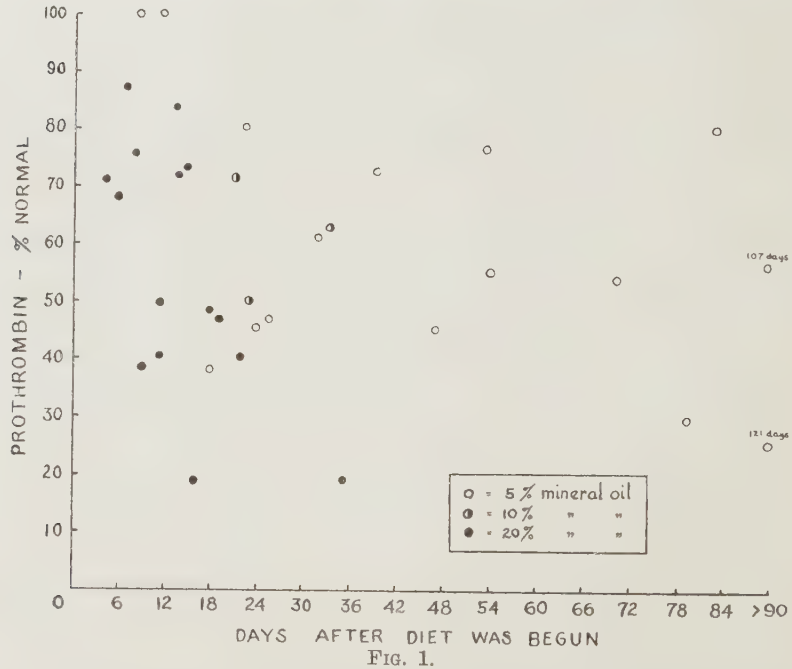


FIG. 1.

Plasma Prothrombin in Mice on a Diet Containing Mineral Oil.

[3] Elliot, M. C., Isaacs, B., and Ivy, A. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 240.

[4] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

Effect of Mineral Oil in the Diet on the Plasma Prothrombin Level. Fig. 1 shows the plasma prothrombin level in mice at various intervals after receiving a diet containing mineral oil. As early as 4 days after mineral oil constituted 20% of the diet, a significant decrease in plasma prothrombin occurred. When 5% of the diet was mineral oil no change was noted until after the 12th day. The individual determinations varied considerably, but after the 3rd week plasma prothrombin levels were usually below 65% of normal. After receiving mineral oil in the diet for several weeks the mice, especially those receiving the large dose of mineral oil, lost varying amounts of hair, appeared poorly nourished, and were smaller than the controls.

Effect of 2-Methyl-1,4-Naphthoquinone on the Plasma Prothrombin Level. Mice with low plasma prothrombin levels were injected intraperitoneally on alternate days for from 2 to 4 times with 0.1 mg of 2-methyl-1,4-naphthoquinone in aqueous solution (0.1 cc). The mice were killed 1 to 2 days after the last injection. Table I shows that the prothrombin level in the blood was elevated to almost normal in most experiments, to normal in one, and to a higher level than the control in another experiment.

Effect of Congo Red on the Plasma Prothrombin Level. Mice with low plasma prothrombin levels, resulting from the addition of mineral oil to the diet, were injected intraperitoneally daily for from 3 to 8 days with 0.1 cc of 1% aqueous solution of Congo red.

TABLE I.

Effect of Intraperitoneal Injection of Congo Red and 2-Methyl-1,4-Naphthoquinone on Plasma Prothrombin Level of Mice on a Diet Containing Mineral Oil.

% mineral oil in diet	Days after feeding mineral oil mice killed	Prothrombin				
		Mineral oil alone	After Congo red		After 2-methyl-1,4-naphthoquinone	
		% normal	% normal	% raised	% normal	% raised
	31	61	76	15	92	31
	39	73	73	0	100	27
5	78	30	35	5	90	60
	83	80	85	5	—	—
	105	57	80	23	92	35
	119	26	57	31	—	—
	12	50	90	40	—	—
	12	40	50	10	—	—
	14	84	96	12	—	—
	14	72	100	28	—	—
20	15	73	84	11	100	27
	18	48	67	19	93	45
	19	47	89	42	100	53
	22	40	100	60	140	100
	22	40	90	50	—	—

The mice were killed on the day after the last injection. Table I shows that plasma prothrombin levels were as much as 60% (average 23%) greater among mice treated with Congo red than among mice on the diet containing mineral oil but which did not receive the chemical.

Two dogs whose prothrombin level had dropped conspicuously following cholecystonephrostomy received Congo red intravenously. In one dog the prothrombin level had dropped to 2% of normal 4 months after cholecystonephrostomy. After the intravenous administration of 5 cc of Congo red (1% aqueous solution) on 6 successive days the plasma prothrombin level rose to 15%. Twenty-four hours after the intravenous injection of aqueous 2-methyl-1,4-naphthoquinone the prothrombin level was 50%. In another dog the prothrombin level was 15% of normal 6 months after cholecystonephrostomy, and following the intravenous administration of 10 cc of Congo red on 2 successive days the plasma prothrombin was 25% of normal.

Discussion. These studies show that in mice prothrombin deficiency can be produced by feeding an adequate diet containing mineral oil, as had been shown previously in rats.[3] With mice, a significant decrease in the level of plasma prothrombin was observed as early as 4 days after mineral oil constituted 20% of the diet. This supports the belief that, unlike most fat soluble vitamins, Vitamin K is not stored by the body in appreciable amounts.[5] A similar rapid decrease in plasma prothrombin has not been observed by others in mammals on a Vitamin K-deficient diet or in mammals with bile excluded from the gastrointestinal tract (*e. g.*, by cholecystonephrostomy or external biliary fistula). Since it has been shown that certain microörganisms, including the colon bacillus, are capable of synthesizing Vitamin K in food, feces or pure culture,[6] it may be that in these animals the bacterial flora of the intestinal tract synthesize sufficient Vitamin K to prevent the rapid decrease in plasma prothrombin. It is possible that in animals with external biliary fistulae or cholecystonephrostomies the addition to the diet of mineral oil or of chemicals that reduce the bacterial flora of the intestine (*e. g.*, sulfaguanidine) might result in a more rapid decline in plasma prothrombin levels. However, the opinion that the inclusion of mineral oil in the diet alone is sufficient to produce a significant change in the prothrombin level of all animals is not supported by a few experiments in which we kept 6 rabbits on a diet containing

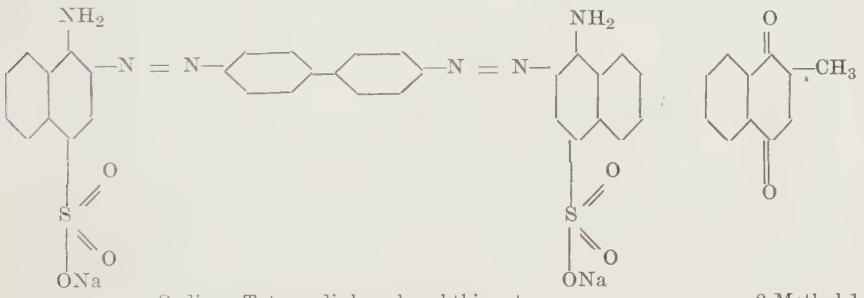
[5] Greaves, J. D., *J. A. M. A.*, 1939, **113**, 389.

[6] Almquist, H. J., Pentler, C. F., and Mecchi, E., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 336.

20% mineral oil (by volume) during a period of 4 weeks with no significant change in the prothrombin level.

The restoration of the prothrombin level to normal or almost normal following the administration of 2-methyl-1,4-naphthoquinone suggested that the diminished prothrombin levels of the mice on a diet containing mineral oil were not due to liver damage[7] but to failure of absorption of Vitamin K.

Many synthetic substances have Vitamin K activity and it is believed that the 1,4-naphthoquinone structure is most essential.[8] However, derivatives of active 1,4-naphthoquinone such as hydroquinones, quinhydrones, hydroquinone esters, or even 1,4-amino naphthols exhibit Vitamin K activity. The structural formula of Congo red is shown below with that of 2-methyl-1,4-naphthoquinone for comparison:



Sodium Tetrazodiphenylnaphthionate (Congo red)

2-Methyl-1,4-naphthoquinone

Most of the human cases in which Congo red presumably diminished or arrested bleeding were apparently not associated with a low prothrombin level,[1] and it is probable that the hemostatic action of Congo red is not confined to its Vitamin K-like effect. However, its beneficial effect on hemorrhage, especially in cases associated with jaundice in humans, may be due, at least in part, to its Vitamin K-like action in raising the plasma prothrombin above the level at which bleeding occurs.

Summary and Conclusions. The level of plasma prothrombin of mice can be lowered by the addition of mineral oil to the diet. The diminished level of plasma prothrombin can be raised significantly by Congo red and restored to normal by the parenteral administration of 2-methyl-1,4-naphthoquinone. The hemostatic action of Congo red may be explained in some cases, at least in part, by its Vitamin K-like action in raising the plasma prothrombin level.

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7 Lord, J. W., and Andrus, W. DeW., *Arch. Surg.*, 1941, **42**, 643; *Arch. Int. Med.*, 1941, **68**, 199.

8 Freeman, S., and Grodius, F. S., *Surg. Gyn. and Obs.*, 1941, **72**, 417.

13449

Intravenous Administration of Bovine Serum Albumin as a Blood Substitute in Experimental Secondary Shock.

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Recent workers[1] have advocated the intravenous administration of whole bovine plasma to human beings. In view of the high incidence of post-transfusion reactions reported by these workers,[1] it is surprising to note that no studies have been reported of the effect of bovine plasma in lower animals. The present paper constitutes preliminary observations upon the responses of dogs to the intravenous injection of bovine serum and plasma and of bovine serum albumin solution.

Material. Healthy dogs weighing from 2.9 to 8.8 kg were used throughout. Bovine serum and bovine plasma were separated from bovine blood by either the gravity or the centrifuge methods. The serum and plasma were then passed through a Seitz filter and stored in a refrigerator at 5°C. In several experiments the serum and plasma were diluted with an equal volume of isotonic saline solution (0.85% sodium chloride). In one experiment, bovine serum was mixed with an equal volume of canine blood. The resultant mixture of bovine and canine serums was separated from the blood cells by centrifugation and passed through a Seitz filter. Bovine serum albumin and bovine serum globulin were prepared by a method which will be described in a later communication together with blood chemical studies. In the present series of experiments the serum albumin obtained from several lots of bovine serum was pooled, and upon analysis revealed an albumin content of 6.7 g %. However, in the final experiment on Table I, the albumin content upon analysis was 4 g %. The bovine serum globulin was obtained from several lots of bovine serum and was pooled. Upon analysis, it revealed a globulin content of 6.4 g %. All the solutions were exposed to room temperature for several hours before being used.

Methods. Anesthesia was induced by nembutal in doses of 1/4 grain per kilo of body weight or by ether given by the open-drop method. The right carotid artery was cannulated and the arterial blood pressure recorded in the usual manner. The plasma, serum and

[1] Wangensteen, O. H., Hall, H., Kremen, A. J., and Stevens, B., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 616.

serum albumin solutions were administered by gravity through a cannula inserted into the right femoral vein. Shock was produced by graded hemorrhage from the carotid artery. Blood was obtained when indicated from an ear of each animal and examined for evidences of agglutination of the red blood cells.

Results. The intravenous administration of whole bovine plasma or serum (150 cc to 400 cc) to normal dogs or to those suffering from hemorrhage resulted in the production of severe dyspnea, the respirations becoming stertorous and labored. The arterial blood pressure rose transiently and then fell gradually to zero. The blood of these animals showed no agglutination of the red blood cells. Dilution of the serum and plasma with equal amounts of isotonic sodium chloride solution did not prevent these toxic effects. Mixture of the serum with equal volumes of canine blood, and reinjection after separation of the blood cells were likewise ineffective. Postmortem examination of these dogs showed the presence of blood stained mucus in the bronchi and subpleural and subserosal petechiae. The spleen was moderately congested. The mucous membranes of the stomach and duodenum exhibited variable degrees of capillary congestion. The behavior of the animals and the absence of agglutination of the blood cells suggested that the fatal effects in these animals might be due to a foreign protein reaction. For this reason it was decided to study the effect of the intravenous administration of bovine serum globulin. This was given in amounts of 125 cc to 155 cc.

Dyspnea similar to that present in the animals which were given whole bovine serum or plasma occurred. The animals appeared to be very sick. The results of the intravenous administration of bovine serum albumin are illustrated in Table I. All of the animals recovered, and showed no obvious ill effects. The arterial blood pres-

TABLE I.
Effect of Intravenous Administration of Bovine Serum Albumin in Secondary Shock. Ether Anesthesia. All Recovered.

Dog wt. kg	Calc. blood vol., cc	Amt blood withdrawn, cc	Amt serum albumin sol. given, cc	Blood pressure		
				Initial, mm Hg	After bleeding, mm Hg	After injection, mm Hg
3.6	324	140	150	140	30	135
5.2	468	160	160	148	15	145
4.8	432	135	135	130	20	123
3.8	342	140	150	140	25	170
5.6	504	180	200	145	40	140
2.8	252	85	80	120	15	100
5.2	468	190	200	150	33	120

sure rose slowly during the injection to the initial level, while in a few animals it rose above the initial level. No disturbances of respiration, such as occurred in the other two groups of dogs, were noted.

Conclusions. Bovine serum, bovine plasma, and bovine serum globulin are very toxic when given by the intravenous route to dogs. The toxicity of the whole serum or plasma appears to be due to the serum globulin or related protein fractions. The intravenous administration of bovine serum albumin is not only harmless, but is effective in raising and maintaining the blood pressure of dogs subjected to severe hemorrhage. Since bovine blood is readily available, bovine serum albumin may prove useful as a substitute for blood. A study of the effects of the intravenous administration of bovine serum albumin in human beings will be reported later.

13450

Effect of Renal and Systemic Bloods from Normotensive and Renal Hypertensive Dogs on Arterial Rings.

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There are several reports claiming to have demonstrated the presence of a pressor substance in the systemic blood of renal hypertensive (Goldblatt) dogs[1, 2, 3] but other investigators have been unsuccessful in similar attempts.[4-9] Obviously this hypothetical pressor substance should be more easily detected in the venous return from the kidney of the renal hypertensive dog. Nevertheless, the reports

1 Govaerts, P., and Dicker, E., *Compt. rend. Soc. de biol.*, 1936, **122**, 809.

2 Solandt, D. Y., Nassim, R., and Cowan, C. R., *Lancet*, 1940, **1**, 873.

3 Page, I. H., *J. Exp. Med.*, 1940, **72**, 301.

4 Page, I. H., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 112.

5 Collins, D. A., and Hoffbauer, F. W., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 539.

6 Friedman, B., and Prinzmetal, M., *Ann. Int. Med.*, 1939, **12**, 1617.

7 Heymans, C., and Bouckaert, J. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 94.

8 Katz, L. N., Friedman, M., Rodbard, S., and Weinstein, W., *Am. Ht. J.*, 1939, **17**, 334.

9 Wakerlin, G. E., and Yanowitz, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 51.

for renal vein blood are about equally divided between positive[3, 10–12] and negative[5, 6, 13] findings.

In a communication already mentioned,[9] one of us reported no significant differences in the vasoconstricting properties for surviving beef arterial rings of systemic plasmas from normotensive and renal hypertensive dogs. Since then we have similarly compared the vasoconstricting effects of renal and systemic bloods from normotensive and renal hypertensive dogs.

The methods employed were essentially those outlined in our earlier report[9] and consisted in recording the contractions of rings obtained from the tertiary division of the superior mesenteric artery of beeves, when heparinized renal vein or femoral artery plasmas or whole bloods were substituted for oxygenated Locke's solution in the smooth muscle chamber. In some experiments, femoral vein blood was also used and in others serum, instead of heparinized plasma or blood, was employed. The renal vein bloods were obtained through previously placed London cannulae, brought out through the anterior abdominal wall. Three dogs were used. After a control period of 10 weekly comparisons between renal vein and femoral artery bloods, the dogs were subjected to bilateral constriction of the renal arteries. The comparisons were then continued during the resulting period of hypertension at weekly intervals for 8, 17, and 34 weeks, respectively. Mean blood pressure readings were obtained by femoral artery puncture 2 or 3 times a week.

As shown by Fig. 1, there were no significant differences between the vasoconstricting effects of the renal vein and femoral artery bloods of the 3 dogs either before or after the production of experimental renal hypertension. There were likewise no significant differences in those experiments in which femoral vein bloods were compared with femoral artery and renal vein bloods, as well as those in which serums were used. The average femoral blood pressures of the 3 dogs during the control period were 139, 129, and 134 mm of Hg, respectively. During the subsequent hypertensive period the average pressures were 183, 176, and 181 mm.

This work, therefore, constitutes another unsuccessful attempt to demonstrate the hypothetical pressor substance of experimental renal hypertension in renal vein blood. However, the limitations of our

10 Braun-Menendez, E., Fasciolo, J. C., Leloir, L. F., and Munoz, J. M., *J. Physiol.*, 1940, **98**, 283.

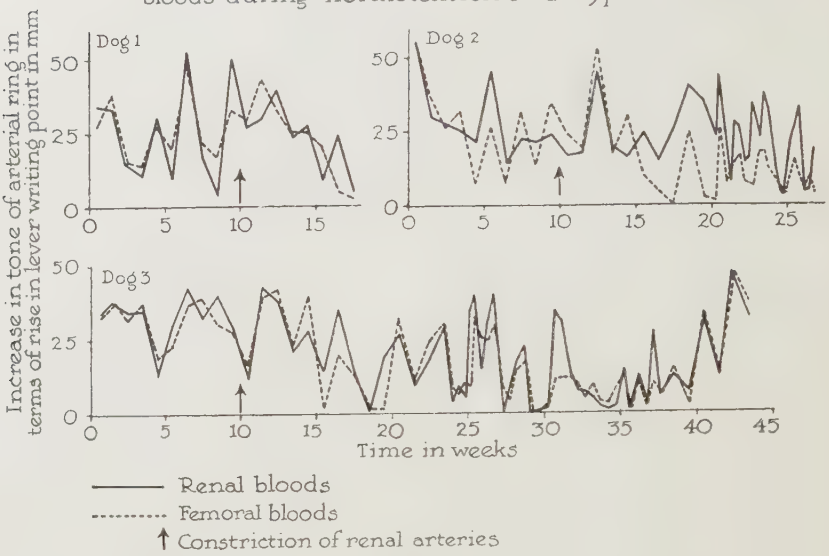
11 Friedman, M., Selzer, A., Sampson, J. J., *Am. J. Physiol.*, 1941, **131**, 799.

12 Goldblatt, H., Kahn, J. R., and Lewis, H. A., *Arch. Surg.*, 1941, **43**, 327.

13 Mason, M. F., and Rozzell, J. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 142.

Fig 1

Tone-augmenting effects of renal vein and femoral artery bloods during normotension and hypertension



method, as previously outlined[9] are such that positive results only would be conclusive.

Conclusions. 1. The vasoconstricting effects of heparinized renal vein and femoral artery bloods from three dogs before and after the production of renal (Goldblatt) hypertension were studied on arterial segments from the mesenteric arteries of beeves. 2. No significant differences were found either during normotension or hypertension. 3. These results do not support the presence of a pressor substance in the renal vein and systemic bloods of Goldblatt dogs but by no means rule out the probability.

13451 P

Changes in Antitryptic Power of the Blood Associated with Anaphylaxis in Guinea Pigs.

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That the natural antitrypsin of body fluids plays a determining

rôle in anaphylactic reactions was postulated many years ago,[1, 2, 3] but the theory has since been almost totally neglected, probably because of the lack of a simple reliable quantitative test for antitrypsin. The present paper records preliminary results of a study in which changes in serum antitrypsin associated with guinea pig anaphylaxis were determined by a new procedure developed by the writer.[4]

Methods and Materials. From a group of 30 male guinea pigs sensitized to egg white blood samples were taken aseptically by heart puncture, without anesthesia, during the incubation period, just before and just after introduction of the shocking dose of the antigen, at death, and, in the case of surviving animals, at various intervals of time after the antigen injections. Normal guinea pigs, inoculated intracardially with egg white as controls, were similarly bled. Blood specimens before and immediately after intracardial antigen injections were obtained in the following way: (1) heart was punctured and the first sample was drawn into the syringe, which was then detached, leaving the needle in the heart, (2) a second syringe containing egg white was connected to the needle and the shocking dose was injected, (3) this syringe was now removed and replaced by a third syringe into which was drawn at once the second sample of blood. Sera collected from the clotted blood specimens were centrifuged until perfectly clear and stored in the refrigerator in sealed sterile tubes until tested.

The antitryptic power of these sera was determined by the film-disc method, following a procedure much simplified and improved over that originally described.[5] Photographic film which has been exposed, developed and hardened in a particular manner is cut into discs 5 mm in diameter. These discs are floated gelatin-side down upon graded dilutions of a standard, stable trypsin solution[6] and upon mixtures of the same trypsin solution in greater concentrations with the serum under test. After incubation at 40°C for an accurately timed period (about 10 minutes) the reactions are stopped, and the dried discs are finally mounted upon a filing card which thus serves as a permanent record. The titer of tryptic activity is expressed in terms of milligrams of trypsin per cc in the dilution which is just strong enough to cause complete clearing of the film (*i. e.*,

1 Bronfenbrenner, J., PROC. SOC. EXP. BIOL. AND MED., 1915-16, **13**, 42.

2 Bronfenbrenner, J., and Schlesinger, M. J., *J. Immunol.*, 1918, **3**, 321.

3 Rusznyak, S., *Deutsch. med. Wchnschr.*, 1912, **38**, 168.

4 A detailed description of the method to be presented elsewhere.

5 Burdon, K. L., and Lafferty, C., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 787.

6 Burdon, K. L., *Science*, 1941, **93**, 91.

TABLE I.

Blood specimens	No. of G.P.	No. of Spec.	Antitryptic index			
			Lower	Higher	Unchanged	Avg
Normal guinea pigs (never inoculated)	8	8				13.6
Normal guinea pigs, 10 sec. to 3 min. after i. c. inoculation of egg white (no reaction)	8	8	1	1	6	13.5
7 days after last of 2 i. p. (sensitizing) inj. of egg white	14	14		V		31.4
14-21 days after last sensitizing inj. and just before inoculation shock dose of egg white	13	13	V			29.6
From sensitized guinea pigs, 10 sec. to 2 min. (avg $<$ 1 min.) after inoculation of shock dose of egg white	15	18	11	2	5	24.6
Just after death from anaphylactic shock (death in 3-5 min.)	14	14	2	3	9	27.5
16 hrs to 5 days after sublethal desensitizing dose of egg white	9	26	2	15	9	35.0

complete digestion of the gelatin layer), and the difference in the titer in the presence and in the absence of a particular serum represents the antitryptic power of that serum. The final figure used to express the *antitryptic index* of a serum sample is obtained by multiplying this difference in titer by the dilution of the serum. For guinea pig sera the dilution factor is 20, since all specimens are diluted 1:20 for the test.

Results. The findings in tests of 101 serum specimens are summarized in Table I.

It will be seen (1) that the antitryptic titer increased markedly as a consequence of the sensitizing doses of egg white, so that at the end of the incubation period the average index was about twice that of the normal, unsensitized animals (a finding not reported previously); (2) that the majority of serum specimens obtained within a minute after intracardial injection of the antigen into *sensitized* guinea pigs showed a decided drop in antitrypsin, while typical symptoms of shock were developing, in contrast to normal animals similarly injected which showed no symptoms and no fall in serum antitrypsin; and (3) that the serum just after death gave an average antitryptic index only slightly higher than the same animals showed before the antigen injection, but surviving guinea pigs had in their sera an especially high content of antitrypsin during the following few days. Several of the latter were shown to be antianaphylactic.

While these data are insufficient to permit final conclusions they demonstrate that definite changes in serum antitrypsin accompany

anaphylactic reactions. They indicate that a sudden drop in antiferment is associated with the initiation of shock, and that a significant increase in antitryptic elements occurs during reactions, so that the serum of surviving, antianaphylactic animals possesses an unusually high antitryptic power.

13452

Evaluation of Bactericides by Egg Injection Method with Special Reference to Development of Technic.*

BERNARD WITLIN. (Introduced by A. D. Welch.)

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Tissue culture methods[1] have been used to measure the toxic action of bactericides through their influence on groups of cells *in vitro*. Such technics, however, do not properly involve the complexity of organ and tissue interrelation which exists in the intact animal, adult or embryonic. The avian embryo was chosen principally for these experiments because manipulation with the whole animal is afforded *in vivo* and because large amounts of albuminous material with which the bactericidal agent might come in contact are present.

General Method. Immediately preceding injection, fertilized eggs of known origin† were candled to assure vitality. The area on the shell opposite the embryo was treated with tincture of iodine and a small hole was drilled‡ through the marked area to expose the *membranae putaminis* (the membrane lining the egg shell). Throughout the manipulation the egg was kept on its side with the marked area uppermost. Using sterile technic, dilutions of the antiseptic made with sterile distilled water were rapidly injected into the egg through a ¼ inch, 27 gauge needle. The hole was then sealed with a drop of sterile paraffin at solidifying temperature.

The eggs were incubated at 38.5°C in an oblique position, with

* Presented before the Pharmacy Subsection of the Medical Division of the American Association for the Advancement of Science, Philadelphia, Pennsylvania, December 28, 1940.

1 Salle, A. J., and Lazarus, A. S., PROC. SOC. EXP. BIOL. AND MED., 1935 and 1936, **32**, 265, 937, 1057, 1119, 1481; **33**, 8, 393; **34**, 371.

† Blood-tested White Leghorns.

‡ Dremel "Moto-Tool" Model No. 1, using burrs No. 107. Manufactured by the Dremel Tool Company, Racine, Wisconsin.

the blunt end up, turned twice daily and then examined for vitality by candling; confirmation of vitality was made by opening the egg.§

Site of Injection. Employing 9-day-old embryos it was found that consistent results were obtained when the medicaments were placed on the chorio-allantoic membrane, whether this was accomplished by the method of Goodpasture,[2] or Brandly,[3] or of Burnett.[4] On the other hand, no degree of consistency was obtained when the injection was made into the albuminous material surrounding the embryonic tissues.

Determining the Volume Capacity of an Embryonic Egg. The injection volume was without influence on the viability of 9-day-old embryos when volumes up to 2.0 cc of saline (0.85%), Tyrode's solution, ethyl alcohol (70%), or F.D.A. broth[5] were injected onto the chorio-allantoic membrane.

Toxicity of Bactericidal Agents. (A) Increasing the Dose by Varying the Volume. A series of 9-day-old fertilized eggs was injected by various methods[2, 3, 4] with stock solutions of the bactericidal agents and their controls. In the first series the volume was increased from 0.1 cc of the medicament to 2.0 cc by 0.1 cc quantities. After preliminary findings were obtained another series was run between the approximate end-points and the sensitivity narrowed down to 0.01 cc. Readings were made after 24 hours' incubation at 38.5°C. Results are shown in Table I.

(B) Increasing the Dose by Varying the Concentration (Volume Constant). Varying amounts of medicaments were diluted to volumes of 2.0 cc with distilled water and injected into 9-day-old avian embryos. Results were obtained identical with those found when the dose was increased by varying the volume.

Bactericidal Efficiency Tests. The bactericidal agents in question were tested by the Food and Drug Administration Technic (F.D.A.).[5] The dilutions of germicide capable of inhibiting the growth of *Staphylococcus aureus*[5] in 10 but not in 5 minutes were noted. All tubes were subcultured to prevent bacteriostatic action by removing one standard loopful[5] from the transplant tube and

§ Vitality is evidenced during candling by movement of the embryo and pulsation of vitelline artery branches. When vitality is doubtful, eggs are opened and the embryo examined for heart-beat.

[2] Goodpasture, E. W., *Southern Med. J.*, 1933, **26**, 418.

[3] Brandly, C. A., *Veterinary Med.*, 1940, **35**, 98.

[4] Burnett, F. M., H. M. Stationery Office Special Report—Series 220 Universal Decimal Classification Number 576, 809, 429:611,013,3/6.

[5] U. S. Food and Drug Administration Bulletin No. 198, 1931.

TABLE I.
Relative Toxicity of Bactericides to 9-day-old Chick-embryos.

Bactericide	Dilution killing *S. aureus* in 10 but not in 5 min. 37°C	A. Actual conc. of bactericide g/cc	9-day chick embryo MLD cc stock soln.	B. grams of bactericide per MLD	$\frac{A}{B} = K$ Toxicity index
Phenol 1:20 aqueous	1:85	.01176	.12	.006	2.000
Hexylresorcinol 1:1000 30% glycerine aqueous solution	1:8,000	.00012	.16	.00016	0.800
Mercuric bichloride U.S.P. 1:1000 aqueous	1:16,000	.00006	.02	.00002	3.125
A mixture of ortho hydroxy phenyl mercuric chloride and 5 isomeric amyl ortho cresols 1:1000 tincture	1:18,000	.00005	.02	.00002	2.750
Sodium hydroxymercuri-o-nitrophenolate 1:1000 aqueous	1:1,100	.0009	.04	.00004	22.750
Sodium ethyl mercuri thiosalicylate 1:1000 tincture	1:3,000	.0003	.02	.00002	16.500
4-nitro-anhydro hydroxy mercuri ortho cresol 1:200 tincture	1:1,100	.0009	.014	.00007	13.000
Disodium dibrom-4-hydroxy mercuri fluorescein 1:50 aqueous	1:12.5*	.080*	.04	.0008	100.000
Iodine, Tincture U.S.P. XI (7% iodine)	1:6430	.00015	.02	.0014	0.11
Sodium hypochlorite solution	1:240	.00416	.04	.0004	10.4
Mild silver protein A 1:20 aqueous	1:100	.010	.9	.045	0.22
Mild silver protein B 1:20 aqueous	1:90	.011	.6	.030	0.366

*Stock solution would not kill test organism under conditions of test.

subculturing into another tube of F.D.A. broth.[5] The results are shown in Tables I and II.

Varying the Age of the Embryo. The effect of the age of the embryo was determined by injecting control fluids (2.0 cc) onto the chorio-allantoic membrane of 12-day-old embryos. In all cases embryos survived after 48 hours' incubation at 38.5°C. Injecting onto the chorio-allantoic membrane by any of the 3 methods.[2, 3, 4] was found to be satisfactory and reproducible results were obtained.

The minimum lethal doses of the germicidal agents for the 12-day-old embryos are recorded in Table II.

Toxicity Index or Coefficient of Relative Toxicity. A comparison was made of the resistance of *Staphylococcus aureus* and of chick-embryos to germicidal agents. The bactericidal efficiencies of the germicides employed were determined by the Food and Drug Administration technic.[5] The highest dilution killing the test organism in 10, but not in 5 minutes, at 37°C after subculturing, was accepted

TABLE II.
Relative Toxicity of Bactericides to 12-day-old Chick-embryos.

Bactericide	Dilution killing *S. aureus* in 10 but not in 5 min 37°C	A. Actual conc. of bactericide g/cc	12-day chick embryo MLD cc stock soln.	B. grams of bactericide per MLD	$\frac{A}{B} = K$ Toxicity index
Phenol 1:20 aqueous	1:85	.01176	.2	.01	1.1764
Hexylresorcinol 1:1000 30% glycerine aqueous solution	1:8,000	.00012	.25	.00025	0.5
Mercuric bichloride U.S.P. 1:1000 aqueous	1:16,000	.00006	.1	.0001	0.625
A mixture of ortho hydroxy phenyl mercuric chloride and 5 isomeric amyl ortho cresols 1:1000 tincture	1:18,000	.00005	.1	.0001	0.55
Sodium hydroxymercuri-o-nitrophenolate 1:1000 aqueous	1:1,100	.00091	.1	.0001	9.1
Sodium ethyl mercuri thiosalicylate 1:1000 tincture	1:3,000	.00033	.1	.0001	3.3
4-nitro-anhydro-hydroxy mercuri ortho cresol 1:200 tincture	1:1,100	.00091	.02	.0001	9.1
Disodium dibrom-4-hydroxy mercuri fluorescein 1:50 aqueous	1:12.5	.08	.3	.006	13.3
Iodine, Tincture U.S.P. XI (7% iodine)	1:6430	.00015	.05	.0035	0.0445
Sodium hypochlorite sol.	1:240	.00416	.3	.003	1.3886
Mild silver protein A 1:20 aqueous	1:100	.010	>2.0*	>0.1*	<0.1
Mild silver protein B 1:20 aqueous	1:90	.011	>2.0*	>0.1*	<0.1

* = No end point with solution used.
> = Greater than.
< = Less than.

as the dilution of the chemical required to kill *Staphylococcus aureus*. The actual amount of germicidal agent present in each cubic centimeter of the highest dilution lethal to the test organism under these conditions was calculated.

The minimum lethal dose (M.L.D.) of the germicidal agents to the embryo of the chick was determined by averaging the results of 3 tests and the actual amount of the germicidal agent present in the minimum lethal dose of the bactericide was calculated.

A number known as the "Toxicity Index" was then determined. It may be defined as the ratio of the actual amount of the germicidal agent contained in each cubic centimeter of the highest dilution required to kill *Staphylococcus aureus* in 10, but not in 5 minutes, at 37°C, to the actual amount of the germicidal agent required to kill a chick-embryo within 24 hours. Theoretically, the smaller the toxicity index the more practical the germicide.

$$\text{Toxicity Index} = \frac{\text{Actual amount of germicidal agent in one cubic centimeter of the highest dilution killing } \textit{Staphylococcus aureus} \text{ in 10, but not in 5 minutes at 37°C}}{\text{Minimal amount of germicidal agent killing the avian-embryo within 24 hours.}}$$

Discussion. The experiments described include an evaluation of toxicity for the chick-embryo with one application of the antiseptic in question. This of course has a valid criticism in looking at the problem from a practical standpoint. In attempting to select an antiseptic on a basis of bactericidal action and relative freedom from tissue toxicity, one must take into consideration either frequent administration of the antiseptic or long continued contact such as is encountered in wet-dressings, irrigations, etc. For this reason both the bactericidal efficiency tests and the embryonic tissue toxicity studies may be considered as acute tests; in addition, tests of a chronic nature would be desirable. The results of such studies might alter the evaluation of the antiseptics studied in this report, particularly with reference to the heavy metals and to the halogens.

Summary. 1. A comparison of various methods for the injection of fertilized and incubated avian eggs has been conducted. The results were similar in all instances wherein the injection was made upon the chorio-allantoic membrane, regardless of the method used to approach the tissue membrane. 2. It was found that large quantities of distilled water, physiological saline, Tyrode's solution and F.D.A. broth could be injected into the egg without affecting the embryo's vitality. Eggs were shown to be capable of sustaining injections of at least 2 cc with maintenance of vitality during at least 48 hours' incubation at 38.5°C following injection. 3. Minimum lethal doses for the avian-embryo were established for the bactericidal agents under test, employing 9- and 12-day-old chick-embryos. The older embryos displayed higher resistance to the toxic substances than did those of 9 days age. 4. Bactericidal efficiencies of the bactericides under test were conducted by the Food and Drug Administration technic, against *Staphylococcus aureus.* In addition, all dilutions of the bactericidal agents were subcultured into another test tube of F.D.A. broth to test for bacteriostasis. 5. A number known as the "Toxicity Index" was determined from a comparison of the bactericidal efficiency against *Staphylococcus aureus,* with the toxicity of the bactericide for chick-embryos.

13453

Effect on Coagulation Time of Oral Administration of Rabbit Thrombin.

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Parfentjev[1] recently described a method for the preparation of pseudoglobulin of rabbit plasma having a high clot-promoting activity. Parfentjev's preparation has been repeated and the activity associated with this pseudoglobulin was found to be thrombic in nature.[2] This was indicated by the ability of the pseudoglobulin fraction to coagulate pure fibrinogen solution in the absence of calcium and to coagulate citrated and oxalated plasma from which prothrombin had been removed. Evidence indicating that this material may have clinical applications as a powerful hemostatic when applied locally to small wounds has also been presented.[3]

The present communication reports the effect of the oral administration of relatively large amounts of pseudoglobulin from rabbit plasma, hereafter referred to as "rabbit thrombin", to dogs, normal subjects and patients suffering from hemophilia.

Methods. Coagulation times were determined on venous blood by the standard procedure formerly described.[4] In certain instances the blood was removed with an oiled syringe and determinations made both in glass tubes and in tubes made of a synthetic plastic "Lusteroid". This procedure prolongs the coagulation time,[5] which makes it possible to evaluate small changes in the coagulation time in glass. In the case of the animal experiments the thrombin was administered either in hamburg steak or milk or in aqueous solution by stomach tube. In the case of human subjects 10 g of the material were dissolved in tomato juice and ingested.

Results. The investigations were carried out on 2 dogs, 2 nor-

* Graduate Fellow of the Belgian-American Educational Foundation 1941-1942.

1 Parfentjev, I. A., *Am. J. Med. Sci.*, 1941, **202**, 578.

2 Taylor, F. H. L., Lozner, E. L., and Adams, M. A., *Am. J. Med. Sci.*, 1941, **202**, 585

3 Lozner, E. L., MacDonald, H., Finland, M., and Taylor, F. H. L., *Am. J. Med. Sci.*, 1941, **202**, 593.

4 Pohle, F. J., and Taylor, F. H. L., *J. Clin. Invest.*, 1937, **16**, 741.

5 Lozner, E. L., and Taylor, F. H. L., *J. Clin. Invest.*, in press.

mal human individuals and 2 patients suffering from hemophilia. When amounts of thrombin varying from 3 to 10 g were given to dogs by mouth there was a prompt fall in the coagulation time, reaching its maximum in about 2 hours, after which it returned toward its initial value. The results obtained in "Lusteroid" tubes paralleled those obtained in glass tubes.

In one normal human subject the results obtained were entirely similar to those found in dogs following oral administration of the material. There was a prompt fall in the coagulation time reaching a minimum in 2 hours with a return toward normal values which was complete in 24 hours. In the second normal subject the initial fall in coagulation time occurred promptly but the return to normal was somewhat delayed.

When 10 g of rabbit thrombin were administered to 2 patients with hemophilia, there was a prompt fall in the coagulation time of the venous blood both in glass and in "Lusteroid" tubes. The data for this observation are given in Table I; they are also illustrative of the effects in both dogs and normal human subjects. In both the hemophilic patients the minimum coagulation time occurred one hour after ingestion and a return to the original prolonged coagulation time had begun in 2 hours.

The results indicate that following the ingestion of large amounts of rabbit thrombin the coagulation time of the circulating blood of dogs, normal human subjects and patients with hemophilia when tested on both glass and Lusteroid tubes is reduced the effect is of short duration.

Comment. The administration of Mill's fibrinogen and McKhann's placenta extract by mouth has been known under certain circumstances to produce a fall in the coagulation time of the circulating blood. These materials are of unknown composition. In rabbit thrombin one has a defined protein which can be administered by mouth and its effect followed in the circulating blood. This phe-

TABLE I.

Effect of Oral Administration of Thrombin on Coagulation Time of Venous Blood of a Patient with Hemophilia in Glass and in "Lusteroid" Tubes.

Subject	Time, hr	Coagulation time, min		Amt. of thrombin administered, g
		Glass	Lusteroid	
	Control	73	125	
			←	10 g in tomato juice
Hemophilia (not fasting)	1	23	40	
	2	27	110	
	3	87	142	
	4	97	120+	

nomenon may have significance to the physiologist interested in the administration of a tagged protein.

So far as the practical use of the administration of rabbit thrombin is concerned, it must be pointed out that relatively large amounts of the material were used. The data indicate that in hemophilic individuals even larger amounts would be necessary to reduce the blood coagulation time to normal. Further studies, however, may produce a more potent material which might result in the production of beneficial effects.

The authors acknowledge the technical assistance of Miss Harriett MacDonald and Miss Marguerite Buckingham.

13454

Metabolism and Food Utilization of Riboflavin-Deficient Chicks.

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Riboflavin deficiency, like most dietary deficiencies, lowers the growth rate of animals. This influence has been described for rats by György and his coworkers[1] and for chicks by Lepkovsky and Jukes.[2] The relation of this stunting of growth to the utilization of food energy and food protein is discussed in this paper.

Method. Five respiration trials of 10 days' duration were carried out with 5 groups of 10 chicks. At the age of 7-9 days the chicks were kept on a diet that was considered deficient in riboflavin, since it led to a stunting of growth and that stunting could be prevented by the addition of riboflavin.

At the age of 12 days 10 chicks in each of the 5 trials were placed in an open-air circulating respiration chamber at a temperature of 29 to 30°C. Food and water were kept before the chicks from 8 a.m. to 8 p.m. During the night the chicks had access only to water. The excreta were collected every 12 hours. Food and excreta were analyzed for nitrogen according to Kjeldahl and for energy in the bomb

* The authors acknowledge the help of Arthur H. Smith, assistant in the Division of Animal Husbandry.

1 György, P., Kuhn, R., and Wagner, Jauregg, T., *Klin. Wochenschr.*, 1933, **12**, 1241.

2 Lepkovsky, S., and Jukes, T. H., *Science*, 1935, **82**, 326.

calorimeter. At the end of the 10 days' feeding period in the respiration chamber the chicks were fasted for 24 hours and then their respiratory exchange was again measured over a 12-hour period during the night. A second similar group of chicks was given the same food as the first but Fuller's earth adsorbate of whey was added as riboflavin supplement. The food intake was restricted to that of the deficient chicks. A third group was kept on the flavin supplemented food *ad lib.*, a fourth group was used as a duplicate of the first and a fifth group as a duplicate of the second. The respiratory exchange during fasting was measured also on 5 extra groups of chicks kept under similar conditions as those mentioned. A mean respiratory quotient of 0.724 indicated proper post-absorptive condition.

To check these results we measured in another group of flavin-deficient and control chicks nitrogen and energy balances by paired feeding and carcass analyses in place of the respiration trials. We used a different flavin-deficient basic diet and crystalline riboflavin instead of Fuller's earth adsorbate of whey as vitamin supplement. The chicks were kept on the flavin-deficient diet for several days. At the age of 20 days they were then separated into 3 equivalent groups according to body weight. Ten chicks were killed at the start of the experiment, 10 were continued on the flavin-deficient food *ad libitum*, and 10 others were supplied each with 0.2 mg of riboflavin dissolved in water and pipetted daily into their crops. The food intake of each of these flavin-supplied chicks was restricted to that of its flavin-deficient pairmate. In 7 pairs we were successful to equalize the food intake closely enough for comparison of the results. The mean daily intake of food energy per chick was 47.9 and 44.2 kcal for the flavin-deficient and flavin supplied chicks respectively. The chicks were kept at 30°C throughout the 10-day experiment, at the end of which they were killed and analyzed for energy and nitrogen.

Results. The most common symptom of a qualitative deficiency in food is a decrease in appetite. Such a decrease resulting from flavin deficiency has been observed in rats by Graham and Griffith.[3] Remp and Bing[4] noted that vitamin G-deficient mice consumed only 60% of the amount of food taken in by vitamin G supplied mice. Riboflavin deficiency similarly decreased the appetite of our chicks. During the first 5-day period of the respiration trials the deficient chicks consumed only ⅔ and during the second period only ½ so much as the flavin supplied controls on unlimited food intake.

[3] Graham, C. E., and Griffith, W. H., *J. Nutr.*, 1933, **6**, 195.

[4] Remp, D. G., and Bing, F. C., *J. Nutr.*, 1934, **8**, 457.

Twenty-nine control chicks, that had been on unlimited intake of flavin-supplemented food weighed at the age of 13 days on the average 71 gm apiece and produced during the subsequent fast a mean of 14.8 ± 1.0 kcal of heat per day per chick or 108 kcal per $kg^{3/4}$.† The corresponding mean daily fasting heat production of 45 flavin-deficient chicks with a mean weight of 51 g amounted to only 10.2 ± 0.8 kcal per chick or 95 ± 6 kcal per $kg^{3/4}$. The same low rate of fasting heat production was, however, observed in 19 controls whose food intake during the 10-day trial had been restricted to that of the deficient chicks, whose mean body weight was 56 g and who produced during fast per day 10.1 ± 1.5 kcal of heat per chick or 96 ± 11 kcal per $kg^{3/4}$. Aside from possible influences of decreased food intake flavin deficiency thus did not affect the rate of fasting heat production in chicks. This result is in line with observations of Drummond and Marrian[5] on vitamin B-deficient rats.

The heat production of the chicks during the 10-day feeding period was calculated on the basis of total carbon and nitrogen balances. The flavin supplied controls on unlimited food intake burnt 75% of the metabolizable energy in their food. The mean heat production of the flavin-deficient chicks amounted to 103% of the metabolizable food energy consumed; that of the flavin-supplied controls on restricted food intake to 109%. Flavin deficiency thus did not produce a greater waste of food energy than did a correspondingly low intake of flavin-supplemented food alone.

Protein utilization in contrast was considerably decreased by flavin deficiency. The flavin supplied chicks on unlimited food intake stored 44% of the food nitrogen in their body, the flavin-deficient chicks as little as 25%. Only one-half of this decrease in total efficiency of nitrogen utilization may be accounted for by the decrease in food intake, since the nitrogen gain of the flavin-supplied chicks on restricted food intake amounted to 35% of the food nitrogen. The difference in the total efficiency of nitrogen utilization between flavin-deficient and flavin-supplied chicks on equal food intake, calculated for 5-day periods, has a random probability of 2% and may therefore be regarded as statistically significant.

Flavin deficiency thus decreased the utilization of protein but did not affect the energy utilization. This observation was confirmed by the pair trials with carcass analysis. The main results of this trial are summarized in Table I.

Since body weight as well as food intake among the pairmates

† $Kg^{3/4}$ is the unit of (body weight raised to the ¾ power).

[5] Drummond, J. C., and Marrian, G. F., *Biochem. J.*, 1926, **20**, 1229.

TABLE I.
Energy and Protein Utilization of Flavin-Deficient Chicks and Pairfed Flavin Supplied Controls.

Flavin in food	Body wt, g	Total efficiency* of utilization of		Mean partial efficiency† of utilization of	
		Energy %	Protein %	Energy %	Protein %
Deficient	121 ± 5	4.8 ± 1.1	9.8 ± 1.6	44.7 ± 1.0	20.1 ± 1.5
Supplied	128 ± 6	5.1 ± 1.0	24.8 ± 2.0	45.7 ± 0.8	35.8 ± 2.0

*Total efficiency $= \frac{\text{gain}}{\text{food}} \times 100.$

†Mean partial efficiency $= \frac{\text{gain} + \text{basal loss}}{\text{food}} \times 100.$

Basal metabolism measured in previous trials.
Basal N loss assumed to be 2 mg N per kcal. basal metabolism.
[Smuts, D. B., *J. Nutr.*, 1935, **9** (4), p. 427.]

were nearly equalized and since the fasting heat production is not affected by flavin deficiency, it is to be expected that the influence of maintenance requirements on total efficiency does not affect the comparisons. Conclusions based on total efficiency should therefore parallel those based on mean partial efficiency which is essentially independent of maintenance requirement. Table I confirms this expectation. Flavin deficiency had no effect on the utilization of food energy but lowered significantly the utilization of nitrogen. For every 100 kcal gain in energy the flavin-deficient chicks gained 2.3 ± 0.7 g nitrogen; their flavin-supplied controls on equal food intake, 5.1 ± 1.4 g. These controls gained protein while losing body fat.

Summary. Respiration trials with groups of flavin-deficient and flavin-supplied chicks as well as pair trials with carcass analysis indicated that flavin deficiency decreased the utilization of protein but did not affect the utilization of food energy beyond the effect of greatly decreased appetite.

13455 P

Cholinesterase in Developing Amblystoma.

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The physiological significance of cholinesterase (ChE) in neuromuscular activity and its importance in the development of behavior

has recently been reviewed by Nachmansohn.[1] He has shown that ChE first appears in appreciable quantities with the beginning of motility in the chick embryo. The concentration of the enzyme at nerve endings is considered high enough to hydrolyze and thereby inactivate, within the refractory period of the contracting muscles, the acetylcholine (ACh) concerned with the neuromuscular transmission. Since the development of behavior pattern in Amblystoma has been so completely elaborated by Coghill,[2] this form seems ideal material on which to study the relationship between enzyme development and behavior. Studies revealing a sharp rise in the esterase content of whole Amblystoma larvae at the time when rapid movements first occur, have already appeared.[3, 4]

The results reported in the present paper represent an extension of this work and an attempt to localize the enzyme throughout ontogeny, *i. e.*, to determine what element or elements in the embryonic neuromuscular apparatus are most active in its production. Direct estimations of enzyme activity were made by means of a modification of Glick's microtitration procedure.[5]

Small amounts of the esterase are present in the premotile embryo where it is already more highly concentrated in nerve and muscle than in other tissues. With the onset of non-tetanic S-flexure responses in the embryo, the concentration of ChE increases to a level more than twice that for the earlier stages. Fig. 1 shows the developmental curves of the esterase in nervous tissue and innervated muscle throughout ontogeny and in nerveless muscle during the prefeeding stages. Both the nerve and innervated muscle esterase curves reach a peak during the early feeding stages and decline through metamorphosis. The innervated muscle curve is similar to that found in the chick by Nachmansohn, who attributes the peak to the higher relative volume of nerve endings during early development. However, Nachmansohn finds no corresponding decline in the chick nerve esterase curve, the adult level being the highest attained. The decline noted in the present work may possibly be caused by the increasing medullation of nerve fibers since myelin contains little ChE. It is significant that the speed of motility and sensitivity to reflex stimulation appear to be greatest during early feeding when ChE content is maximal, and that the marked retarda-

[1] Nachmansohn, D., *Yale J. Biol. and Med.*, 1940, **12**, 565.

[2] Coghill, G. E., *Anatomy and the Problem of Behavior*, 1929, Cambridge.

[3] Youngstrom, K., *J. Neurophysiol.*, 1938, **1**, 357.

[4] Sawyer, C. H., *Anat. Rec.*, 1940, **78**, Supp. 57.

[5] Glick, D., *J. Gen. Physiol.*, 1938, **21**, 289.

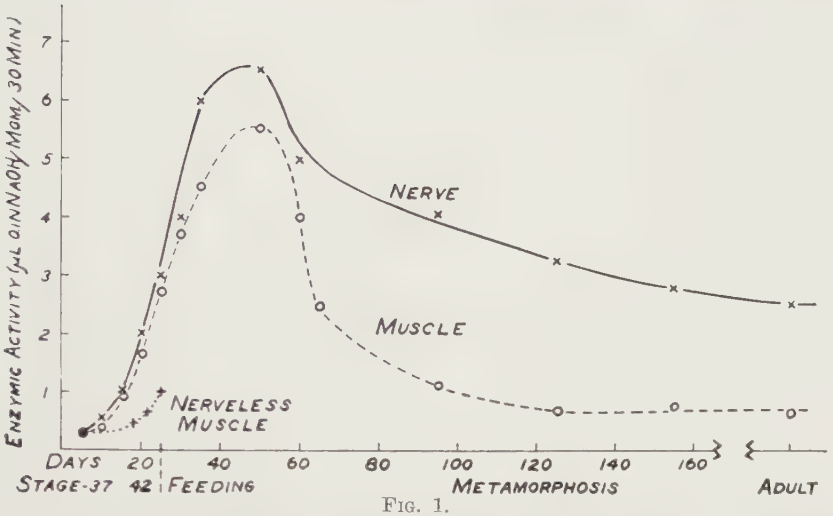

FIG. 1.

Development of cholinesterase in nervous tissue, innervated muscle, and nerveless muscle of *Amblystoma punctatum*. Each point represents the average of several determinations.

tion of movement and increased threshold to stimuli during metamorphosis can be correlated with decreased enzyme content.

The peaks in nerve and muscle esterase curves occur at the period when, according to Wills,[6] the respiratory rate of developing Amblystoma is highest. This relationship may well be causal; increased ChE permitting more rapid movements which in turn lead to a higher metabolic rate.

There is disagreement as to the effect of denervation on the content of muscle esterase. Martini and Torda[7] find a considerable decline of esterase activity in denervated muscle of rats and dogs, but Couteaux and Nachmansohn[8] report that the concentrations of enzyme (Q_{ChE}) are equal in denervated and innervated guinea pig gastrocnemius. To determine the part played by nerve endings in the development of ChE in embryonic muscle, nerveless muscle has been produced by removing the spinal cord from embryos at an early premotile stage. The resulting muscle differs from ordinary denervated muscle in that it has never been under the influence of a functional nervous system. It is at first responsive to direct stimuli but later fails to respond to direct induction coil shocks. Enzyme determinations (Fig. 1) indicate that the amount of esterase in this

6 Wills, I. A., *J. Exp. Zool.*, 1936, **73**, 481.

7 Martini, E., and Torda, C., *Klin. Wochensch.*, 1938, **17**, 97.

8 Couteaux, R., and Nachmansohn, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 177.

nerveless muscle is about a third of that in muscle with nerves. Thus it appears that the bulk of ChE production can be ascribed to nerve endings. These findings, in harmony with those of Martini and Torda, are further links in the chain of evidence coupling cholinesterase and neural activity.

To summarize, cholinesterase in the embryo appears to be concentrated at nerve endings and to be directly related to speed of motility and to metabolic rate. It seems clear that cholinesterase content is a biochemical criterion of functional capacity in the developing neuromuscular apparatus.

13456

Phosphate Equilibrium Between Plasma and Saliva.*

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Incidental to a trial of radioactive phosphorus in the therapy of carcinomatous metastases to bone we have been able, through the courtesy of Dr. George E. Fahr, to make some observations as to the secretion of radiophosphorus in the saliva. The results have some bearing on the interpretation of the studies by Sognnaes and Volker[1] and Barnum and Armstrong[2] dealing with the uptake of radioactive phosphorus by dental enamel from the saliva. The subject was a woman, aged 52, with advanced carcinoma of the breast, and the observations were made 19 days before death when the patient was not cachectic. Four grams of sodium phosphate, containing 42,000,000 Geiger-Mueller counts of radioactive phosphorus, dissolved in 100 cc of water were administered by stomach tube.

One hour following the administration of the radioactive salt a sample of saliva, stimulated by the chewing of paraffin, was collected over a 26-minute period. A second saliva sample was collected 3 hours after administration of the salt and, midway in this collection, a sample of venous blood was obtained. The final saliva sample

* This work was carried out with the aid of a grant from the Carnegie Corporation. The radiophosphorus used in this work was kindly supplied by Professor S. K. Allison of the University of Chicago.

1 Sognnaes, R. F., and Volker, J. F., *Am. J. Physiol.*, 1941, **133**, 112.

2 Barnum, C. P., and Armstrong, W. D., *Am. J. Physiol.*, in press.

was taken 24 hours after the administration. The urine was collected during the 24 hours following the administration of the active phosphate.

The saliva samples were centrifuged to remove the mucus, treated with equal volumes of 14% trichloracetic acid and centrifuged again. The supernatant fluid was used for the determination of radiophosphorus and total phosphorus. The blood was collected in an oxalate tube and centrifuged. The proteins were removed from the plasma by addition of trichloracetic acid to 5% concentration and the supernatant fluid was used for the active phosphorus and chemical phosphorus determinations. The radioactivity measurements were made upon basic calcium phosphate precipitates prepared under identical conditions by a technic developed in this laboratory.

Table I shows the phosphorus content of saliva and plasma and also the specific activities (percent of administered radiophosphorus found per mg of phosphorus) of the fluids. Equality of distribution of phosphate between 2 fluids is demoted by equality of values for specific activity. Since the specific activities of the first 2 saliva samples and the point for zero activity, when plotted against time, fell on the same straight line it appears that the peak of the saliva activity was reached somewhere between 3 and 24 hours. The 3-hour saliva activity failed to reach a value equal to the plasma activity. A possible explanation for the failure to find the expected equilibrium between plasma and saliva might be that inactive phosphorus, stored in the salivary glands, was secreted with the active phosphorus derived from the plasma. It is also possible that absorption of phosphate from the intestine had not been completed 3 hours after the administration of the salt so that the specific activity of the plasma was still increasing when the blood was drawn.

The urine was treated with an excess of calcium lactate and made alkaline. The calcium phosphate precipitate was ashed and dissolved in HNO_3 and the phosphate isolated by the method of Hull and Williams.[3] The recovered radiophosphorus amounted to 15.8% of that administered and it was used in some further studies. Since some of the radiophosphorus was lost during the various steps in-

TABLE I.
Distribution of Phosphorus between Plasma and Saliva.

	mg P/cc	Sp. Act. (×1000)
1 hr saliva	0.196	2.75
3 " "	0.195	7.49
24 " "	0.162	4.74
Plasma (inorganic phos.)	0.054	17.6

[3] Hull, D. E., and Williams, J. H., *Rev. Sci. Instr.*, 1940, **11**, 299.

volved in the isolation, this result means that a minimum of 16% of the administered dose was lost in the urine in the first 24 hours. This extreme clinical inefficiency might be overcome by a preliminary period of phosphorus starvation since it has been observed in this laboratory that rats on a high calcium-low phosphorus diet reduce their urinary output of radioactive phosphorus to about 1/60th of its normal value. If such a diet were employed preliminary to the clinical use of radiophosphorus it would be necessary to administer the active phosphorus by a parenteral route or, if the oral route is used, to subject the patient to a preliminary fast sufficiently long to clear the intestine of calcium.

13457

Experimental Production of Target Cells by Splenectomy and Interference with Splenic Circulation.*

EDWARD B. MILLER, KARL SINGER AND WILLIAM DAMESHEK.
(Introduced by J. H. Pratt.)

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Target cells are erythrocytes which in the stained blood film show a central mass of hemoglobin within an unstained intermediate zone which is in turn surrounded by a peripheral rim of hemoglobin, thus giving the appearance of a "bull's eye" or target. Haden and Evans[1] first described these cells as "Mexican hat" cells and stated they were characteristic of sickle cell anemia. Barrett,[2] who suggested the term "target cell", found them in increased numbers in obstructive jaundice, certain cases of hypochromic anemia, in steatorrhea, and following splenectomy. He also demonstrated that target cells were abnormally thin and showed an increased hypotonic resistance. Recently these cells were described by Dameshek[3] and by Wintrobe, *et al.*,[4] as the outstanding hematological feature of a

* Aided by grants from the Charlton Fund, Tufts College Medical School, and the Dazian Foundation.

1 Haden, R. L., and Evans, F. D., *Arch. Int. Med.*, 1937, **60**, 133.

2 Barrett, A. M., *J. Path. and Bact.*, 1938, **46**, 603.

3 Dameshek, W., *Am. J. Med. Sci.*, 1940, **200**, 445.

4 Wintrobe, M. M., Matthews, E., Pollack, R., and Dobyns, B. M., *J. A. M. A.*, 1940, **114**, 1530.

hitherto undescribed type of hereditary anemia probably related to Cooley's anemia and found in adult Italians.

The occurrence of target cells following splenectomy, and in sickle cell anemia in which the spleen not infrequently becomes atrophic, points to a possible relationship of these cells to splenic function. In a previous study,[5] target cells were almost regularly demonstrated (unless spherocytosis persisted) in 19 patients splenectomized for various conditions. It was evident that these cells might remain in considerable numbers for several years after removal of the spleen. In the present study, an attempt has been made to produce target cells experimentally either by splenectomy or by interference with the splenic blood supply.

Methods. Splenectomy was performed in dogs, guinea pigs and rabbits. Interference with the splenic circulation was performed in dogs and consisted either of almost complete elimination of the arterial blood supply, or of complete ligation of all the splenic veins with the exception of some anastomoses with the gastric vessels. In a few experiments incomplete ligation of the splenic veins was performed. Determinations of the hemoglobin, red cell count, white cell count, platelet count, hypotonic (sodium chloride) fragility, and of the percentages of target cells and Howell-Jolly bodies (among 1000 erythrocytes) were made. In the animals studied, target cells were either lacking or present in small numbers (less than 1%); Howell-Jolly bodies were not normally present.

Results. Dogs. Large numbers of target cells were regularly observed after splenectomy in 3 dogs. In "Yellow Dog," which was studied for 162 days after splenectomy, the number of target cells remained below 20% during the first 2 months but increased later (109 to 134 days) to levels between 40 and 70%. There was an associated change in the "minimum" value of the hypotonic fragility, from .26 to .12%, indicating an increased resistance of certain cells, later followed by a change in the "maximal" fragility, from .56 to .46%. Howell-Jolly bodies increased concomitantly with the target cells.

Complete ligation of the splenic veins in 2 dogs likewise led to a considerable increase of target cells, which was accompanied by a slight increase in the hypotonic resistance. Howell-Jolly bodies were, however, lacking or found only in very small numbers.

Incomplete obstruction of the splenic veins was also followed by the development of target cells but in considerably smaller numbers than following the other operations. Ligation of the splenic artery in

[5] Singer, K., Miller, E. B., and Dameshek, W., *Am. J. Med. Sci.*, 1941, **202**, 171.

2 dogs was followed by a slight inconstant increase of target cells. Howell-Jolly bodies were lacking.

Guinea Pigs. Splenectomy in 6 guinea pigs was followed by the development of target cells but in considerably smaller numbers than in dogs, the highest value encountered being little more than 10%. There was also a concomitant decrease of the minimum saline fragility and the appearance of Howell-Jolly bodies.

Rabbits. Splenectomy in 3 rabbits failed to result in target cells or in a change of the hypotonic fragility during periods of observations up to 120 days. Howell-Jolly bodies were, however, observed.

Discussion. The appearance of considerable numbers of target cells following both splenectomy and complete splenic vein ligation point either to a direct influence of the spleen upon the erythrocytes passing through it or to a hormonal effect upon the production of red cells in the bone-marrow. It is conceivable that the red cells upon their production in the bone-marrow are relatively thin (target cells) and develop a "normal" degree of thickness in the process of passage through the sinusoids of the spleen. Numerous studies[6] bearing directly or indirectly on the problem, indicate that the red blood cells

TABLE I.
"Yellow" Dog.
Ligation of Splenic Artery; Splenectomy.

Date	Days preligation	R.B.C. (millions)	Hgb. %	W.B.C.	Target cells %	Howell-Jolly bodies %	NaCl fragility %
	33	4.0	69	19,000	0	0	.56-.26
	31	3.9	64	19,200	0	0	—
	16	4.2	71	19,000	0	0	.48-.28
			Ligation of Splenic Artery.				
	Postligation						
11/8/39	2	4.1	63	24,600	0	0	.44-.24
11/16	10	4.6	73	21,300	1.0	0	.46-.28
11/23	17	3.8	61	29,000	4.4	0	.52-.22
			Splenectomy.				
	Postsplenectomy						
12/1	0	4.3	67	48,200	2.6	0	.50-.34
12/22	21	4.9	72	25,500	6.3	.3	.44-.16
1/4/40	33	4.3	75	—	8.0	.4	.50-.20
1/16	45	4.7	76	19,900	18.0	.6	.42-.20
1/30	59	4.0	71	19,200	16.7	.4	.50-.12
3/20	109	4.8	84	12,300	72	.7	.44-.16
3/29	118	4.4	82	—	40.8	1.2	.46-.20
4/15	134	5.3	86	—	50.6	.9	.46-.26
4/24	143	5.0	75	18,400	31.4	.5	.42-.22
5/13	162	4.6	81	20,300	36.2	.8	.46-.24

[6] Ham, T. H., and Castle, W. B., *Proc. Am. Philos. Soc.*, 1940, **82**, 411; Knisely, M. H., (a) *Anat. Rec.*, 1936, **65**, 23; (b) *Ibid.*, p. 131; Lauda, E., *Die normale und pathologische Physiologie der Milz*, Urban & Schwarzenberg, 1933.

TABLE II.
Guinea Pig No. 80.
Splenectomy.

Days presplenectomy	R.B.C. (millions)	Hgb. %	W.B.C.	Platelets (×000)	Target cells %	Howell-Jolly bodies %	NaCl fragility %
23	5.6	96	13,100	1,845		0.0	.40-.24
16	5.2	80	15,100	1,967	0.2	0.0	.42-.16
			Splenectomy.				
Postsplenectomy							
7	4.3	71	10,800	2,812	1.8	1.5	.44-.16
32	5.2	90	29,200	2,021	1.8	1.5	.42-.20
34	4.6	65	29,900	1,829	7.0	0.9	—
39	4.3	73	24,500	1,450	7.7	1.9	—
						Norm 1	
41	4.3	71	23,300	2,888	8.0	1.9	—
						Norm 1	
63	5.3	78	24,900	2,421	5.3	0.7	—
70	5.0	77	18,800	1,355	10.6	0.4	.34-.04
80	5.3	80	23,100	—	8.6	0.3	.34-.12

become modified in their passage through the spleen and that the modification is in the direction of increased spheroidicity and increased hypotonic fragility. In the absence of the spleen, it is likely that the red cell population tends on the whole to be thinner than normal, a definite proportion of erythrocytes becoming sufficiently thin to be recognized as target cells. It is likewise possible that the spleen exerts some influence upon the last stages of erythrocyte maturation and that in the absence or diminution of such a hypothetical splenic maturation principle target cells may develop. Such a hormonal influence seems probable as Hirschfeld and Weinert[7] first suggested, in the explanation of the development of Howell-Jolly bodies.

Summary. Target cells (abnormally thin erythrocytes) appeared in considerable numbers following splenectomy in dogs and guinea pigs and complete ligation of the splenic veins in dogs. In rabbits, splenectomy failed to result in the development of target cells. The spleen either has a direct effect on the red cells passing through it or a hormonal effect on the last stages of erythroblastic maturation in the bone-marrow.

[7] Hirschfeld, H., and Weinert, H., *B. klin. Wschr.*, 1914, **22**, 1026.

13458 P

Filth Flies as Transmitters of *Endamoeba histolytica.*

Alan C. Pipkin. (Introduced by E. C. Faust.)

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During the last 25 years, epidemiological studies[1, 2] have thrown suspicion on flies as possible transmitters of amebiasis. Wenyon and O'Connor,[3] using the eosin viability test, found cysts remained viable as long as 24 hours in the gut of flies. Root[4] showed that cysts apparently remained viable as long as 49 hours after ingestion by flies as judged by the neutral red test.

Due to doubt concerning the absolute reliability of such staining technics as criteria of viability, the present investigation was designed to prove or disprove, by modern culture technics, the viability of cysts or trophozoites of *E. histolytica* washed from the external body surfaces or recovered from or passed through the alimentary tract of flies.

The 5 species of filth flies utilized in these experiments were *Sarcophaga misera, Phormia regina, Cochliomyia macellaria, Lucilia pallescens,* and *Musca domestica.* Cysts used were concentrated by the zinc sulfate technic of Faust, Tobie, *et al.,*[5] and collected by the D.C.F. method of Lane,[6] thoroughly washed and stored in physiological saline at ice-box temperatures. The trophozoites were cultivated on the Frye and Meleney[7] modification of the original Boeck and Drbohlav coagulated egg medium. Cultures were incubated at 37°C and transplanted every 48 hours.

To test the effect of external carriage on the subsequent viability of trophozoites and cysts, 60 flies of each species were contaminated with feces enriched with cultured trophozoites and feces seeded with cyst concentrates. Over a period of 10 minutes, flies were removed from the exposure cage in groups of 3 at 30-second intervals and washed, and the washings inoculated into culture media.

1 Craig, C. F., *The Military Surgeon,* 1917, **40**, 286.

2 Frye, W. W., and Meleney, H. E., *Am. J. Hygiene,* 1932, **16**, 729.

3 Wenyon, C. M., and O'Connor, F. W., *Human Intestinal Protozoa in the Near East* (Monograph), pp. 218, 1917, London and New York.

4 Root, F. M., *Am. J. Hyg.,* 1921, **1**, 131.

5 Faust, E. C., Tobie, J., *et al., Am. J. Trop. Med.,* 1938, **18**, 169.

6 Lane, C., *Trans. Roy. Soc. Trop. Med. and Hyg.,* Part I, 1923, **16**, 274.

7 Frye, W. W., and Meleney, H. E., *Science,* 1939, **89**, 564.

Controls consisted of the cultivation of washings from 3 flies immediately following contamination. Trophozoites were found to be culturable after periods of from ½ minute (for *L. pallescens*) to 1½ minutes (*S. misera* and *P. regina*) of external exposure. Cysts were found to be culturable after periods of from 1 minute (*M. domestica*) to 4 minutes (*S. misera*) of external exposure.

To obtain some indication of the survival of trophozoites and cysts at different levels of the alimentary tract, the following experiments were performed. Groups of from 15 to 20 flies of the designated species were fed singly on cultured trophozoites or on feces seeded with cyst concentrates. Flies fed on trophozoites were killed and examined at 5-minute intervals up through 1½ hours, while those fed on cysts were sacrificed at 30-minute intervals up through 6 hours. Controls consisted of the cultivation of the same trophozoite and cyst material which was fed to the flies.

Trophozoites were found culturable after remaining in the crop for periods of from 15 minutes (*M. domestica* and *L. pallescens*) to 40 minutes (*S. misera*), and after remaining in the gut for periods of from 5 minutes (*M. domestica, L. pallescens,* and *P. regina*) to 30 minutes (*S. misera*). Motile trophozoites were not recovered from the recta of any of the 5 species. Cysts were found to be culturable after remaining in the crop for periods of from 20 minutes (*L. pallescens*) to 210 minutes (*S. misera*), however all species of flies other than *S. misera* gave values below 30-minute maxima. Cysts were found to be culturable after remaining in the gut for periods of from 20 minutes (*L. pallescens*) to 240 minutes (*M. domestica*) and were found to be culturable after remaining in the rectum for periods of from 30 minutes (*P. regina*) to 180 minutes (*S. misera*).

To test the viability of cysts and trophozoites deposited in the vomitus and fecal droplets of flies, the following experiments were performed. Groups of 15 flies of each species were fed separately on cultured trophozoites or feces seeded with cyst concentrates. Vomitus and fecal droplets were cultured immediately after deposition. The cyst experiments have been repeated several times for each species to add significance to the results. To date a total of 375 flies of the 5 species have been tested. Controls consisted of the cultivation of the same trophozoite and cyst material fed to the flies.

It was found that trophozoites recovered from the vomitus were culturable after periods of from 9 minutes (*C. macellaria*) to 17 minutes (*L. pallescens*) of internal carriage. Cysts were culturable from the vomitus after periods of from 28 minutes (*C. macellaria*)

to 64 minutes *(P. regina)* of internal carriage. Cysts were found culturable from fecal droplets for periods of from 137 minutes *(P. regina)* to 218 minutes *(C. macellaria)*.

Conclusions. From the evidence cited above, it seems highly probable that the filth flies studied play no important rôle as transmitters of trophozoites or cysts of *E. histolytica* by external carriage. However, the viability of cysts for periods as long as 3½ hours after initial ingestion and later dejection indicates a potential natural method of transmission.

13459

Carbohydrates of the Gonadotropic Hormones.

SAMUEL GURIN. (Introduced by D. Wright Wilson.)

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The presence of carbohydrate in various purified gonadotropic hormone preparations raises a number of questions concerning the significance of the carbohydrate and its relationhip to biological activity. Evans, *et al.*, have studied the carbohydrate of various pituitary[1, 2] as well as pregnant mare serum preparations.[3] McShan and Meyer[4] have recently called attention to the possible significance of carbohydrate in their studies of pituitary follicle stimulating hormone preparations. The nature of the carbohydrate in the gonadotropic hormone of urine of pregnancy has likewise been studied.[5]

The data reported by Evans, *et al.*,[2] indicate that the interstitial cell stimulating hormone of the pituitary contains mannose, while a preparation made from pregnant mares' serum was found to contain galactose. Studies along these lines have been carried out in this laboratory and a comparison of the carbohydrate in various gonadotropic hormones is reported here.

The carbazole method[6] was employed for the identification and

1 Evans, H. M., Fraenkel-Conrat, H., Simpson, M. E., and Li, C. H., *Science*, 1939, **89**, 249.

2 Li, C. H., Simpson, M. E., and Evans, H. M., *Endocrinology*, 1940, **27**, 803.

3 Li, C. H., Evans, H. M., and Wonder, D. H., *J. Gen. Physiol.*, 1940, **23**, 733.

4 McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, 1940, **135**, 473.

5 Gurin, S., Bachman, C., and Wilson, D. W., *J. Biol. Chem.*, 1940, **133**, 467.

6 Gurin, S., and Hood, D. B., *J. Biol. Chem.*, 1939, **131**, 211.

TABLE I.

Hormone	Concentration mg/cc	ϵ540	ϵ520/ϵ420	Hexose present	Hexosamine %	Hexose %	Hexose / Hexosamine
Pituitary L. H.	1.901	.159	0.66	Mannose	2.8	2.2	1.27
" F. S. H.	0.998	.130	0.73§	"	4.5	4.4	1.02
Equine Gonadotropin*	1.270	.627	1.00	Galactose	15.6	8.3	1.88
" " †	1.430	.755	1.10	"	17.6	8.4	2.10
Human Pregnancy Urine Gonadotropin			0.95-1.05	"	10-12	5-6	2
Non-Pregnant Mare Serum‡	1.100	.307	0.80	Mannose-Galactose	10.0		

*Preparation No. 18-5-1; assay 3300 R.U./mg. For a discussion of the biological assay method see reference 11.

†Preparation No. 15-111-1; assayed 4000 R.U./mg.

‡Kindly supplied by Dr. H. Goss.

§This value is slightly higher than the one usually obtained with pure mannose. It suggests the presence of a small amount of another hexose.

estimation of the sugars. The Palmer-Smyth-Meyer[7] modification of the Elson-Morgan procedure[8] was employed in the estimation of hexosamine after preliminary hydrolysis in sealed tubes with 4 N HCl for 8 hours at 95-100°. The results are summarized in Table I.

The luteinizing and follicle-stimulating hormones prepared from pituitary glands were generously supplied by Dr. H. B. van Dyke. They represent highly purified fractions prepared by the method of Greep, van Dyke and Chow.[9] It will be observed that the ratios ϵ520/ϵ420 indicate that mannose is the sole or main hexose present in these preparations. The molecular ratios of hexose to hexosamine are in both instances close to unity indicating that, in these preparations, the carbohydrate is composed of mannose and hexosamine in equimolar proportions. These results are in harmony with the findings reported by Evans[2] for the carbohydrate of the interstitial cell stimulating hormone. The total carbohydrate in these fractions is somewhat lower, however, than the values obtained by Evans.

Two samples of gonadotropic hormone obtained from pregnant mare serum were kindly furnished by Dr. H. Goss. These preparations were made by the method described by Goss and Cole.[10] The ϵ520/ϵ420 ratios obtained with these samples indicate that the hexose present is either galactose or an equimolar mixture of glucose and mannose. The carbohydrate in these preparations appears to contain hexose and hexosamine in a molar ratio of 2:1.

7 Palmer, J. W., Smyth, E. M., and Meyer, K., *J. Biol. Chem.*, 1937, **119**, 491.

8 Elson, L. A., and Morgan, W. T. J., *Biochem. J.*, 1933, **27**, 1824.

9 Greep, R. O., van Dyke, H. B., and Chow, B. F., *J. Biol. Chem.*, 1940, **133**, 289.

10 Goss, H., and Cole, H. H., *Endocrinology*, 1940, **26**, 244.

11 Cole, H. H., and Erway, J., *Endocrinology*, 1941, **29**, 514.

It is probable that the hexose is actually galactose in view of the findings of Evans[2, 3] although there is some discrepancy concerning the ratio of hexose to hexosamine. This may probably be ascribed to the greater purity of our preparations.

A preparation made from non-pregnant mare serum by a similar procedure proved to contain equimolar amounts of mannose and galactose. In this case the carbohydrate moiety appears to be similar to that present in serum mucoid and in other serum glycoproteins.

It is interesting to note that the carbohydrate groups of the gonadotropins of pregnant mare serum and of urine of pregnancy in women are apparently similar and that they are different from those found in the pituitary gonadotropic hormones. It is difficult to believe that these results are purely fortuitous and that the presence of a different type of carbohydrate in the gonadotropins associated with pregnancy (gestation) is not a result of the marked metabolic changes occurring during pregnancy.

Summary. A qualitative as well as a quantitative study has been made of the hexose present in the gonadotropic hormones of the pituitary gland, pregnant mare serum and human pregnancy urine. The hormones obtained from the pituitary gland contain mannose and hexosamine in equimolar proportions. The gonadotropins of pregnant mare serum and human pregnancy urine appear to contain galactose rather than mannose. In these preparations the molar ratio of hexose to hexosamine is 2:1.

13460 P

Influence of Estrogen on Respiration of Rat Uterine Tissue.

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Although much is known about the morphological changes that take place in various tissues and organs due to the influence of the different sex hormones, little is known about true physiological effects, and still less about the actual biochemical action of the active substances. Hence an investigation has been undertaken to locate the mode and place of action in a physiological system of one of the female sex hormones, estradiol.

One of the physiological effects of estrogen, and one that lends itself to biochemical analysis, is the increase in oxygen consumption

of the uterus. There have been several investigations of the respiration of mouse and rat uteri,[1–5] and all of these agree that estrogen increases the oxygen consumption. Kerly,[4] and Büngler and Erhardt[2] showed that the anaerobic glycolysis of uterine tissue is increased by injection of estrogen, and the latter authors also found an increase in the aerobic glycolysis.

The technics used in the present study differ in several respects from those of previous studies. First, a pure estrogen was used. This was estradiol dipropionate, 50 μg of which is sufficient to produce a continuous estrous effect for at least a month.[6] Second, effects of a continuous treatment with estrogen were determined. Third, measurements were made on separate strips of endometrium and muscle. Fourth, dry weights were determined on other uterine tissue treated in the same way as that used for the respiration measurements. Summerson manometers were employed for the determination of oxygen consumption, aerobic glycolysis, and R. Q.

The changes in the oxygen consumption of uterine tissue with time after injection of estradiol dipropionate follow a peculiar curve. The normal value of the Q_{O_2} for uteri from castrated, uninjected animals is 3.9. There are only slight increases at 6 and 12 hours after injection, but at 24 hours the Q_{O_2} has reached 6.15. However, the peak, 8.0, is reached by 45 hours and is maintained until about 75 hours, when the Q_{O_2} falls off to a plateau of 6.6. This level is maintained for several weeks, or as long as the oestrous effect of the injection lasts. The values given for tissue after 24 hours' injection are for the endometrium. The Q_{O_2} values of muscle follow the same sort of curve but are slightly smaller: 6.65 at the peak, and 6.3 for the final plateau.

It may be significant that the values of the aerobic glycolysis ($Q_G^{O_2}$) reach their peak and then their final plateau much sooner than those of the Q_{O_2}. The normal value is 1.5; the peak comes at 24 hours and is 3.1 for the endometrium and 2.0 for muscle. The drop to the final plateau begins immediately and by 70 hours has reached 1.0 for endometrium and 0.5 for muscle.

R. Q. values increase from 0.89 in the normal untreated controls to 1.0 at 24 hours, but soon fall again to about 0.90 by 70 hours.

1 David, J. C., *J. Pharmacol.*, 1931, **43**, 1.

2 Büngeler, W., and Erhardt, K., *Klin. Wchnschr.*, 1931, **10**, 593.

3 Khayyal, M. A., and Scott, C. M., *Quart. J. Exp. Physiol.*, 1935, **25**, 77.

4 Kerly, M., *Biochem. J.*, 1940, **34**, 814.

5 MacLeod, J., and Reynolds, S. R. M., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 666.

6 Miescher, K., Scholz, C., and Tschopp, E., *Biochem. J.*, 1938, **32**, 725.

Glucose has the expected effect of raising the R. Q. in all cases except those of uterine muscle from animals injected for more than 70 hours. In these cases it is lowered slightly.

A more noticeable influence of glucose is the inhibitory effect it has on the Q_{O_2} of both endometrium and muscle from the 24-hour injected animals. The inhibition averaged about 20%. After 24 hours, oxygen consumption was practically the same with and without glucose.

The values of the anaerobic glycolysis follow a pattern similar to those for the changes in oxygen consumption. The average value for uteri from untreated animals is 6.1 ($Q_G^{N_2}$), the peak at 60 hours is 11.5, and the plateau at 90 hours and beyond is about 9.25.

These results show the direction and magnitude of the effect which estrogen has on the respiratory metabolism of uterine tissue. A further analysis to find the point of action of estrogen in the respiratory cycle should be possible, perhaps with the aid of inhibitors of specific respiratory enzymes or enzyme systems.

13461 P

Encephalitis Virus "Antibody" in Sera of Experimentally Infected Animals by Agglutination of Virus-Coated Cells.

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Details of the technic involved in satisfactorily coating bacterial cells with encephalitis virus and the agglutination of such cells by the sera of encephalitis patients have been given in a previous report[1] wherein the test was designated as the bacterial agglutination (B.A.) method of detecting serum antibody. That encephalitis virus was actually adsorbed onto the bacteria was indicated by the infectivity of 'coated' cells for susceptible animals even after repeated washing to remove extraneous virus. The specific nature of the agglutination reaction with patients' serum was demonstrated by certain control tests wherein the reaction did not occur when non-coated cells or cells coated with normal mouse brain were used. Further, sera from cases of clinically recognized poliomyelitis were studied which did not agglutinate cells coated with encephalitis virus although they were

[1] Roberts, E. C., and Jones, L. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 75.

capable of agglutinating in high titer poliomyelitis virus-coated cells. And further, a high specificity was indicated by the ability of mouse-brain containing encephalitis virus as well as bacterial cells coated with this material to remove the agglutinating factor from the sera of encephalitis patients; while on the other hand, normal mouse brain or bacterial cells coated with normal mouse brain failed to remove the factor.

The application of this method to the detection of 'antibodies' in the blood serum of monkeys and hamsters experimentally infected with the virus of St. Louis encephalitis is the basis of the present report.

In Table I are given the results of the application of this method to the sera of hamsters wherein it will be noted that positive B.A. tests have been observed as early as the second day following inoculation with the virus of St. Louis encephalitis. Since this agglutinating factor has not been observed in the serum of normal animals, we presume that the B.A. titer of 1:10 observed in the case of animal No. 11 is related to the inoculation of virus 2 days previously. In the case of hamster No. 14, which survived for a considerable period of time, positive B.A. reactions with significant serum titers persisted for several weeks.

In Table II are recorded the results observed in the sera of 2 monkeys experimentally infected with St. Louis encephalitis virus. Serum samples taken from these animals before inoculation failed

TABLE I.

Serum Titers, Based upon Agglutination of Virus-coated Bacteria (the B. A. Method), in Hamsters Inoculated with St. Louis Encephalitis Virus.*

Hamster No.	Route of inoculation	Days, post-inoculation	B.A. titer
1	Normal	Control	Neg.
2	"	"	"
3	Intracerebral	4	64
4	"	4	128
5	"	5	Neg.
6	"	19	16
7	Intranasal	5	Neg.
8	"	5	32
9	"	6	32
10	"	7	64
11	Subcutaneous	2	10
12	"	6	32
13	"	8	20
14	"	12	64
"	"	42	32
"	"	49	64
"	"	56	64
"	"	70	32

*Serum samples supplied by Dr. G. O. Broun.

TABLE II.

Serum Titers, Based upon Agglutination of Virus-coated Bacteria (B.A. Method), in Monkeys *(M. rhesus)* Inoculated with St. Louis Encephalitis Virus.*

	B.A. SL Enceph. virus	Temp. °F	Symptoms	B.A. SL Enceph. virus	Temp. °F	Symptoms
Pre-inoculation	Neg.	102.6	—	Neg.	104.0	—
	(Intracerebral Inoculation)			(Intranasal Inoculation)		
Post-inoculation, days						
2	32	101.8	—	32	103.0	—
5	8	103.4	+	16	104.0	—
7	8	106.0	++++	64	104.6	—
8	32	105.0	++++			
9	16	105.6	++	32	104.0	+
11	16	102.4	+	64	105.3	++++
13		103.6	+	32	104.6	++++
19		103.8	—	32	104.0	—

*Serum samples supplied by Dr. G. O. Broun.

to elicit the B.A. reaction while serum samples obtained 48 hours post-inoculation were capable of agglutinating encephalitis virus-coated cells. Later samples taken during the course of the disease showed a significant rise in titer. The agglutinating factor appeared 4 days prior to the onset of symptoms in the case of the animal inoculated by the intracerebral route, and 7 days prior to symptoms in the case of the intranasally inoculated animal.

The early appearance of an agglutinating factor in the experimentally infected animal, which can be detected by this very sensitive technic, suggests the possible application of the method as a diagnostic aid in neurotropic virus infection of man and it is conceivable that a particular merit of the method may be its ability to detect the factor very early in the course of the disease.

13462 P

Influence of Vitamin E (d-l-Alpha Tocopherol Acetate) on Blood Cholesterol and Fatty Acids of Male Schizophrenics.*

RALPH ROSSEN AND AARON REICHENBERG. (Introduced by Ancel Keys.)

From the Research Division, Hastings State Hospital, Hastings, Minn.

The demonstration by Adamstone[1] of a marked decrease in brain cholesterol in chicks with encephalomalacia on a diet deficient in Vitamin E (d-l-alpha tocopherol) suggested the present study of the influence of large doses of Vitamin E on the blood cholesterol and fatty acids of 8 adult male patients with schizophrenia.

Experiment. Patients on bed rest were given a daily diet of 200 g of CHO, 63 g of P and 53 g of F, yielding 1500-1600 calories, during the study period and for 2 weeks prior to the use of large doses of Vitamin E. Two fasting blood samples were taken from the cubital veins, a day apart, just before the ingestion of Vitamin E, and single specimens were obtained thereafter at 7-10-day intervals for 77 days. The quantitative determination of cholesterol and fatty acids was made by the methods of Lieberman-Burchard[2] and Bloor[3] respectively.

The administration of Vitamin E was discontinued for 3 periods during the study in order to detect any changes during temporary discontinuance of Vitamin E. During the 77-day period, 5345 mg of Vitamin E were given *per os* as follows: 570 mg during the first 11 days; discontinued 7 days; 2425 mg the next 25 days; discontinued for 13 days; 2350 mg the next 16 days and then discontinued for the last 5 days. One hundred fifty-two days later the same patients were given the same diets except that no Vitamin E was administered and control blood studies were made at weekly intervals for 70 days.

Results. Blood cholesterol and fatty acids values are shown on Table I. The trend of values for cholesterol during the experimental period shows little variation. At the end of the first week on Vitamin E a 10% increase was noted over the mean value of all controls taken

* The Hastings State Hospital wishes to state its sincere appreciation to the Hoffman LaRoche Co., for supplying gratis the Vitamin E that was used in this investigation.

1 Adamstone, F. B., *Arch. Path.*, 1941, **31**, 711.

2 Evelyn Photoelectric Colorimeter, Bulletin 46, p. 32 (Modified Lieberman-Burchard Reaction).

3 Bloor, W. E., *J. Biol. Chem.*, 1916, **24**, 447.

TABLE I.

The following values show the blood cholesterol (C*) and fatty acids (FA†) in mg per 100 ml at weekly intervals on each patient while he received Vit. E; as compared with a like period of time when he served as a control.

Patient		1st wk‡		2nd wk		3rd wk		4th wk		5th wk		6th wk		7th wk		8th wk	
		C*	FA†	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA	C	FA
No. 1	On Vit. E	150	352	165	420	140	390	175	395	188	385	178	408	162	340	160	348
	Control	145	315	152	322	142	360	148	360	158	332	153	340	158	352	153	322
No. 2	On Vit. E	182	440	232	536	240	560	238	510	242	540	192	530	215	450	213	444
	Control	195	360	194	398	192	365	178	365	176	363	176	375	172	355	172	370
No. 3	On Vit. E	122	328	162	410	128	342	138	344	152	372	174	382	136	374	132	365
	Control	155	345	160	355	148	373	158	355	162	358	160	335	160	345	168	330
No. 4	On Vit. E	182	460	225	525	185	525	205	508	215	484	215	466	174	442	178	430
	Control	210	400	215	395	200	370	210	390	200	375	200	360	188	384	§	§
No. 5	On Vit. E	162	425	180	452	162	440	160	390	168	385	158	360	152	385	166	382
	Control	‖															
No. 6	On Vit. E	135	390	158	400	172	408	220	510	168	485	184	442	162	395	182	404
	Control	172	355	176	363	168	320	166	365	172	330	162	340	160	355	155	340
No. 7	On Vit. E	140	315	162	380	134	370	132	330	144	340	146	348	122	320	126	335
	Control	132	310	136	315	128	325	142	338	136	320	124	330	120	334	128	322
No. 8	On Vit. E	172	485	196	462	172	450	160	420	196	370	160	380	162	375	176	395
	Control	190	370	188	358	187	358	190	370	195	365	192	390	196	380	192	368

C*—Cholesterol.
FA†—Fatty acids.
‡First cholesterol and Fatty acid (under first week) are values before Vit. E administered.
§On home visit.
‖No control series—patient on home visit.

over a 70-day period. After the first week, there was a marked increase of over 25% of the fatty acids values while on Vitamin E as compared with the mean fatty acids value of the controls taken over the entire control period. The fatty acids values for the patients on Vitamin E decreased gradually over the experimental period until there was a difference of only 5% as compared with the mean value of the entire control period. This study indicates that large doses of Vitamin E cause a primary increase in fatty acid content of the blood of male patients with schizophrenia. Studies are being carried on at this time to determine the cause and significance of these changes.

13463 P

Dermatitis in Pyridoxine-Deficient Rats.

Samuel Lepkovsky and Myrtise E. Krause. (Introduced by H. J. Almquist.)

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Pyridoxine deficiency in rats has always been associated with a "specific dermatitis" which has been named acrodynia.[1] Dermatitis can be readily produced when thiamine and riboflavine are the only members of the Vitamin B complex fed.[2, 3] While such a supplemented diet has often been referred to as a pyridoxine-deficient diet, it is deficient also in factor 2 or the filtrate factors. Factor 2 concentrates are now known to carry pantothenic acid plus other as yet unidentified filtrate factors. Addition of factor 2 will sometimes promote the development of dermatitis[4] and sometimes will give erratic results.[5]

In this laboratory for a long time no dermatitis appeared on large numbers of pyridoxine-deficient rats, though the rats were obviously deficient since they either did not grow at all or grew slowly and responded spectacularly to pyridoxine administration. Because a good deal of dermatitis had been observed on pyridoxine-deficient rats in the past, the records were searched for changes in technic which might account for the absence of dermatitis on pyridoxine-deficient rats. The absence of dermatitis was found to be coincident with a change in technic involving feeding of supplements to the basal diet. In previous work, 21-day-old rats were put on the synthetic diet[4] without any addition of water-soluble vitamins for 10 to 12 days. Thiamine and riboflavine were then added for 14 days after which factor 2 concentrates[4] were added. A large proportion of these rats developed dermatitis. The change in technic consisted in feeding thiamine, riboflavine and factor 2 concentrates to the rats when they were put on the synthetic diet at 21 days of age without any depletion period. Such rats, though they developed pyridoxine deficiency, seldom developed dermatitis.

Forty rats were accordingly put on the basal diet[4] for a depletion period of 12 days after which, for a period of 2 weeks, only thiamine

1 Birch, T. W., György, P., and Harris, L. J., *Biochem. J.*, 1935, **29**, 2830.

2 György, P., *Biochem. J.*, 1935, **29**, 741.

3 Antopol, W., and Unna, K., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 126.

4 Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, 1936, **115**, 557.

5 Dann, W. J., *J. Biol. Chem.*, 1939, **128**, XVIII.

and riboflavine were fed. At this time symptoms of dermatitis began to appear. Factor 2 concentrates were added, and definite dermatitis appeared in a week. In 2 weeks, 16 of the rats developed severe dermatitis, 15 mild, and 12 rats showed no dermatitis. After 5 weeks of factor 2 feeding, only 4 rats remained without dermatitis. Three rats with slight dermatitis had cured spontaneously.

It thus appears, at least from these experiments, that dermatitis develops as a result of a factor 2 deficiency superimposed upon a pyridoxine deficiency. If rats are early exposed to such an extra deficiency, the effects, insofar as they affect dermatitis, seem to carry over after the addition of factor 2 concentrates, and the rats become deficient only in pyridoxine. The question may well be raised whether dermatitis or acrodynia is a reliable expression of uncomplicated pyridoxine deficiency.

13464

Experimental Necrotizing Arteritis. II. Mercuric Chloride as Effective as Uranium Nitrate in its Production.*

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In a recent publication[1] the senior author described well marked necrotizing arterial lesions affecting principally the large elastic arteries (aorta, sinuses of Valsalva, endocardium of the left auricle, coronary and pulmonary arteries) which appeared unexpectedly during the course of the experiments designed to determine whether heavy metal poisoning is influenced by altering the plasma protein level.

Normal adult dogs maintained on a standard low protein diet were made hyperproteinemic by repeated intravenous injections of plasma obtained from healthy donor dogs. Each dog then received a single subcutaneous injection of 5.0 mg of uranium nitrate per kilo. In 4 of the 5 dogs in which this procedure was carried out, acute necrotizing arterial lesions were found at necropsy 8-17 days after the injection of the heavy metal; and healed lesions were found when the 5th dog

* This investigation was aided by a grant from the John and Mary R. Markle Foundation.

[1] Holman, Russell L., *Am. J. Path.*, 1941, **17**, 359.

was sacrificed 11 months after the administration of uranium nitrate.

No definite conclusions were reached about the pathogenesis of the lesions but the suggestion was offered that the lesions "may be due to a hypersensitivity to some constituent of the blood of one or more of the donor dogs precipitated by uranium nitrate or made manifest in the disturbed metabolism consequent to the uranium nitrate injury."

In this paper are described the results of using mercuric chloride instead of uranium nitrate. Only 2 dogs have been tried but both yielded positive results showing that corrosive sublimate can be substituted for uranium nitrate in the production of these arterial lesions.

The methods were the same as those employed in the previous study, the only difference being that mercuric chloride (3.0 mg/kg *intravenously*) was used instead of uranyl nitrate (5.0 mg/kg *subcutaneously*). The standard basal diet consisted of: calves' liver (raw wet weight) 32 parts, cane sugar 25 parts, corn starch 25 parts, butter 12 parts, and cod liver oil 6 parts. Enough tomato juice was added to make a paste of which each gram contained 3 calories. One gram of McCollum-Simmonds salt mixture[2] and 5 g of kaolin were thoroughly mixed with each day's diet. The diet was fed in amounts to furnish 75 calories per kg per day.

The plasma injections used in making the dogs hyperproteinemic were carried out as follows: About 200 cc was bled from a healthy adult donor dog into a flask containing 2.5 cc of saturated solution of sodium citrate. The citrated blood was centrifugalized for 20-30 minutes at 3000 rpm in 100 cc centrifuge tubes. The plasma was then drawn off with suction, warmed to 40-45°C and injected into the jugular vein. Approximately 10 minutes was required for the plasma (usually 90-110 cc) to run into the vein. This procedure was repeated 6 times per week for 3-4 weeks before the heavy metal was injected.

The mercuric chloride was obtained from J. T. Baker Co. (lot 6939) and was injected to the jugular vein in 0.1% aqueous solution. Sufficient water was used to insure quantitative transfer.

Blood plasma protein and non-protein-nitrogen were determined by duplicate micro-Kjeldahl analyses. Trichloracetic acid (10%) was used to precipitate the protein for the determination of non-protein-nitrogen.

The dogs were necropsied promptly after death. Sections from all organs and from many of the tissues were stained routinely with

[2] McCollum, E. V., and Simmonds, N., *J. Biol. Chem.*, 1918, **33**, 55.

TABLE I.
Summary of Experimental Data.

Dog No.	No. of inj.	Total amt plasma inj. cc	Plasma protein concentration Before inj. g/100 cc	Plasma protein concentration After inj. g/100 cc	Body wt.* kg	Mercuric chloride inj. intrav. mg/kg	Height of non-protein nitrogen† mg/100 cc	Survival interval‡ days
40-63	24	2960	6.0	8.0	9.2	3.0	394	7
40-80	18	1840	6.7	9.4	5.8	3.0	466	6

*Body weight at end of period of plasma injections, *i.e.*, weight on date of mercuric chloride injection.

†In dog 40-63 the terminal blood sample was taken 2 hours before death; in dog 40-80, 3 hours before death.

‡Interval between injection and autopsy.

TABLE II.
Anatomical Distribution of Arterial Lesions.*

Dog No.	Ascending aorta	Sinuses of valsalva	Left auricle	Pulmonary artery	Arteries in lungs	Other arteries
40-63	+++	++	+++	+++	+†	
40-80	++†	++	+++	+++†	+	Innominate ++† (aneurysm)

*The lesions have been graded as follows: +++ = gross lesion over 1 cm in maximum diameter; ++ = gross lesion less than 1 cm in maximum diameter; + = lesion discovered in microscopic section.

†Thrombus associated with lesion.

——— Microscopic illustration.

hematoxylin and eosin. Special stains used in selected cases were: Verhoeff's for elastic tissue, Kossa's for calcium and VanGiesson's or Mallory's for the differentiation between muscle and connective tissue.

Table I summarizes the experimental data and Table II indicates the anatomical distribution of the arterial lesions. The cause of death in both dogs was presumably "uremia". The figures for the terminal non-protein-nitrogen and the extensive necrosis of the proximal convoluted tubules seen in histological sections of the kidneys substantiate this clinical impression.

The arterial lesions are similar in all respects to those previously reported with uranium nitrate.[1] Essentially the lesion is an acute necrotizing arteritis usually most marked in the intima but not infrequently involving all 3 coats. Polymorphonuclear neutrophils predominate in the cellular response. Hemorrhage and fibrin deposition are not infrequent. Sometimes thromboses occur. Typical lesions are illustrated in Fig. 1.

One lesion deserves special comment. A small saccular aneurysm about 8 mm in diameter and hemispheric in shape was found at the mouth of the innominate artery in dog 40-80. This aneurysmal bulge was partly filled with laminated thrombus and there was a slight

FIG. 1.

Dog 40-80. 6 days after mercuric chloride. × 110. Sections stained with hematoxylin and eosin.

A. Acute necrotizing auriculitis. Leukocytic infiltration and necrosis beneath intact endocardium.

B. Acute necrotizing arteritis of pulmonary aorta with thrombosis and aneurysmal outpouching.

amount of hemorrhage in the adventitia, but no definite rupture. Sections of the wall stained with Verhoeff's method show almost complete destruction of the elastic framework of the media. In

other respects this lesion resembles those previously described. This is the first aneurysm that has been observed in this series, but the possibility was suggested by the fragmentation and disintegration of elastic tissue seen in previous lesions.[1]

Thus the observations with uranium nitrate have been confirmed with mercuric chloride. Experiments are now in progress to determine whether the heavy metal as such or the renal insufficiency following the administration of the heavy metal is the essential factor in the production of these experimental arterial lesions. The other two factors apparently concerned in their production are diet and plasma alteration.

Summary. Mercuric chloride can be substituted for uranium nitrate in the production of necrotizing arterial lesions in dogs. An aneurysm developing on the basis of one of these lesions is described for the first time.

13465

Epithelial Hyperplasia of Hassall's Bodies of Thymus Gland Induced by Methylcholanthrene.

PAUL E. STEINER. (Introduced by Paul R. Cannon.)

From the Department of Pathology of the University of Chicago.

The histogenesis and functions of the thymus gland are still imperfectly understood. Uncertainty exists concerning the essential nature of Hassall's bodies, the thymic reticulum, and the thymic lymphoid cells. The rôle of the thymus as a gland of internal secretion, and as a source of lymphocytes is also not clear.

During experiments designed to induce tumors in the thymus with a cancerogenic hydrocarbon observations were made which give support to the idea that Hassall's bodies are epithelial and not endothelial, and that some of the thymic reticulum may be epithelial in nature.

Procedure. Cylindrical pellets consisting of 10 mg of methylcholanthrene plus 10 mg of cholesterol and measuring approximately 1.75 mm in diameter and 8-10 mm in length were[1] implanted into the thymus gland of young guinea pigs of both sexes by open surgical operation.

Animals were sacrificed at 7, 14, 21, and 28 days and irregularly at

[1] Shear, M. J., *Am. J. Cancer*, 1936, **26**, 322.

other earlier and later time intervals. The left lobe of thymus which contained the pellet was fixed in Zenker's solution or in formalin, as was the corresponding right unoperated lobe. They were embedded in paraffin after which sub-serial sections were cut. The sections were stained by hematoxylin and eosin, by Mallory's aniline blue method, and in critical instances also by Dr. George Gomori with his modification of Mallory's phosphotungstic acid-hematoxylin method[2] to demonstrate intercellular bridges. Thus the pellet space could be studied at numerous levels to be sure that the representative cellular reactions were seen. The pellets themselves were dissolved away by the alcohol and the xylol used in making the preparations.

Results. Fibrous encapsulation of the pellets, which were apparently unchanged in size, was already present at 7 days and subsequently the connective tissue became more mature and abundant. There was a leukocytic reaction to the pellets as well as sometimes a foreign body giant cell reaction.

The changes in the thymic tissue consisted of acute degeneration of the thymic lymphocytes, irregular areas of hyperplasia of thymic reticulum, and hyperplasia of Hassall's bodies all in the region within about 0.5 to 2.0 mm of the pellets, epithelialization of the pellet space, and epithelial cyst formation.

The hyperplasia of Hassall's bodies was seen as early as the 7th day. They enlarged and their centers became cystic, the cysts containing cell debris including the centers of the old Hassall's bodies.

Most remarkable was the epithelialization, by a stratified squamous type of epithelium, of the pellet space. This was first seen at 14 days. Its origin in one case, studied in serial section, and illustrated in Fig. 2, was from a Hassall's body which sent an epithelial stalk toward the pellet space and, reaching it, spread to line the space. In other animals this relationship was not seen, and the possibility is not excluded that the epithelium came from other thymic cells.

The encapsulated space occupied by the pellet sometimes enlarged up to 1 cm in the first month. This additional space contained fluid, leukocytes and cell debris. In other instances similar cyst-like spaces were found in close proximity to the pellet space and communicating with it through a small opening revealed by serial sections. In still other instances no such connection was seen and the impression given was that the cysts were enlarged Hassall's bodies.

A marked hyperplasia of cells was sometimes seen in the regions near the pellet in which the small, dark thymic cells had disappeared. These cells were large, plump spindle to polyhedral with a large

[2] Gomori, G., *Am. J. Path.*, 1941, **17**, 395.

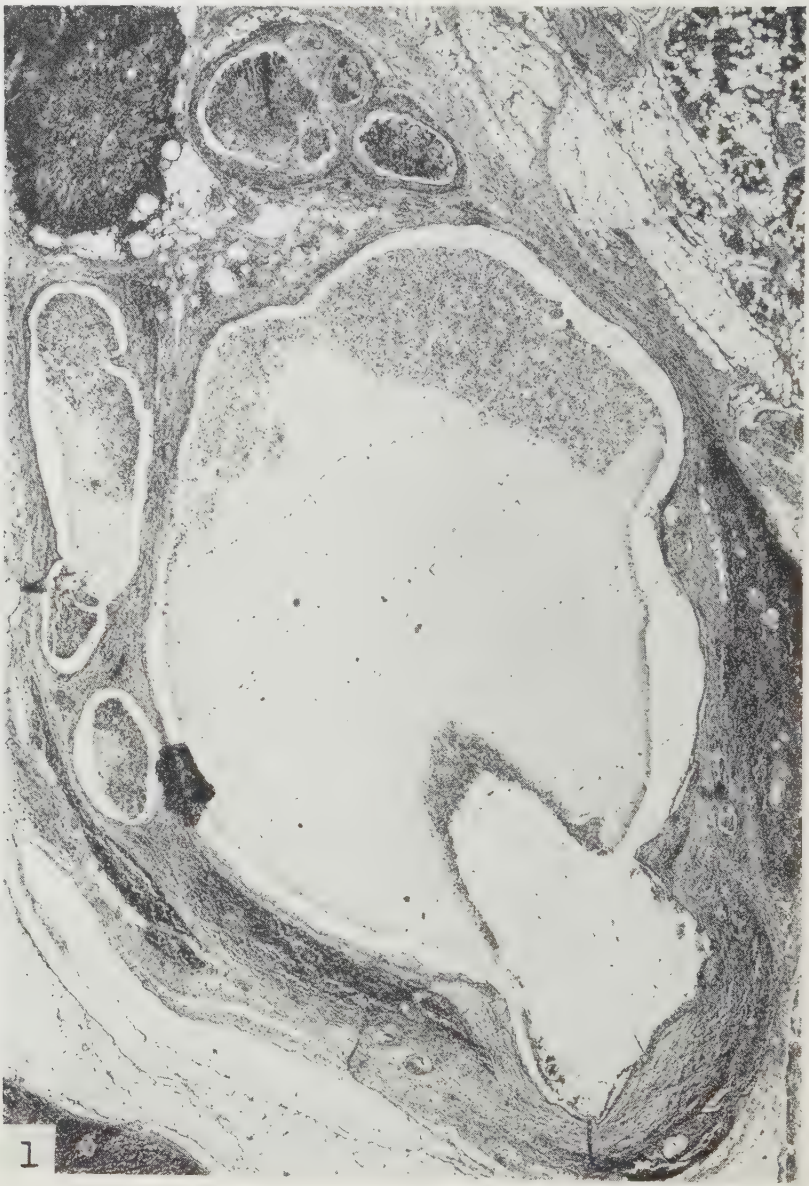

FIG. 1.
(× 25) The pellet has been dissolved from the lower right. Its space is enlarged and is lined by stratified squamous epithelium. At the left and above are independent squamous cysts resembling enlarged forms of Fig. 3.

oval nucleus. Such regions showed a marked paucity of intercellular fibrils, and they sometimes merged gradually with definite

stratified squamous epithelium lining the pellet space. These cells appear to have had their origin from thymic reticulum, and they appear to be forming squamous epithelium.

Epithelial proliferation seemed to take place at a slow rate after the first month, some keratinized material slowly accumulating in the cysts. After about one year palpable masses were present which sometimes grew rapidly. These were true tumors and will be described elsewhere.

Controls. Controls were studied to establish the normal structure of the thymus for guinea pigs having the same age, sex, environment, and operative procedure. They consisted (a) of guinea pigs who were implanted with pellets of the same size but composed of 20 mg of cholesterol, (b) of guinea pigs with pellets of methylcholanthrene and cholesterol implanted in other tissues and organs also located in the neck (salivary glands, muscle, adipose tissue, and lymph nodes), and (c) of the contralateral, unimplanted thymic lobes from the experimental animals.

Similar changes were not seen under any of these conditions. Epithelialization of the space occupied by the pellet was seen only in implants into the salivary gland as previously reported.[3] The inflammatory reaction and the fibrous response appeared to be quantitatively less when cholesterol alone was used.

Comment. These hyperplasias are of interest because they precede neoplasias and the latter are generally considered to be difficult to produce in guinea pigs, especially epithelial tumors.[4, 5] Methylcholanthrene, like some other chemical cancerogens can have destructive actions on cells and it is also of interest that the concentration required to induce the epithelial proliferation was approximately the same as that capable of destroying the small thymic lymphocyte-like cells.

The new cells produced in Hassall's bodies, in the cyst linings, and in the lining of the pellet spaces are stratified squamous epithelium as is shown by their general morphology, intercellular bridges, and the formation of keratohyaline. Whether they represent the process of hyperplasia or of metaplasia, they indicate that Hassall's bodies are epithelial.

Ross and Korenchevosky[6] have reported epithelial hyperplasia and

3 Steiner, Paul E., *Third International Cancer Congress*, page 138, Sept., 1939.

4 Warren, Shields, and Gates, Olive, *Cancer Research*, 1941, **1**, 65.

5 Shimkin, Michael B., and Mider, G. Burroughs, *J. Nat. Canc. Inst.*, 1941, **1**, 707.

6 Ross, M. A., and Korenchevosky, V., *J. Path. and Bact.*, 1941, **52**, 349.

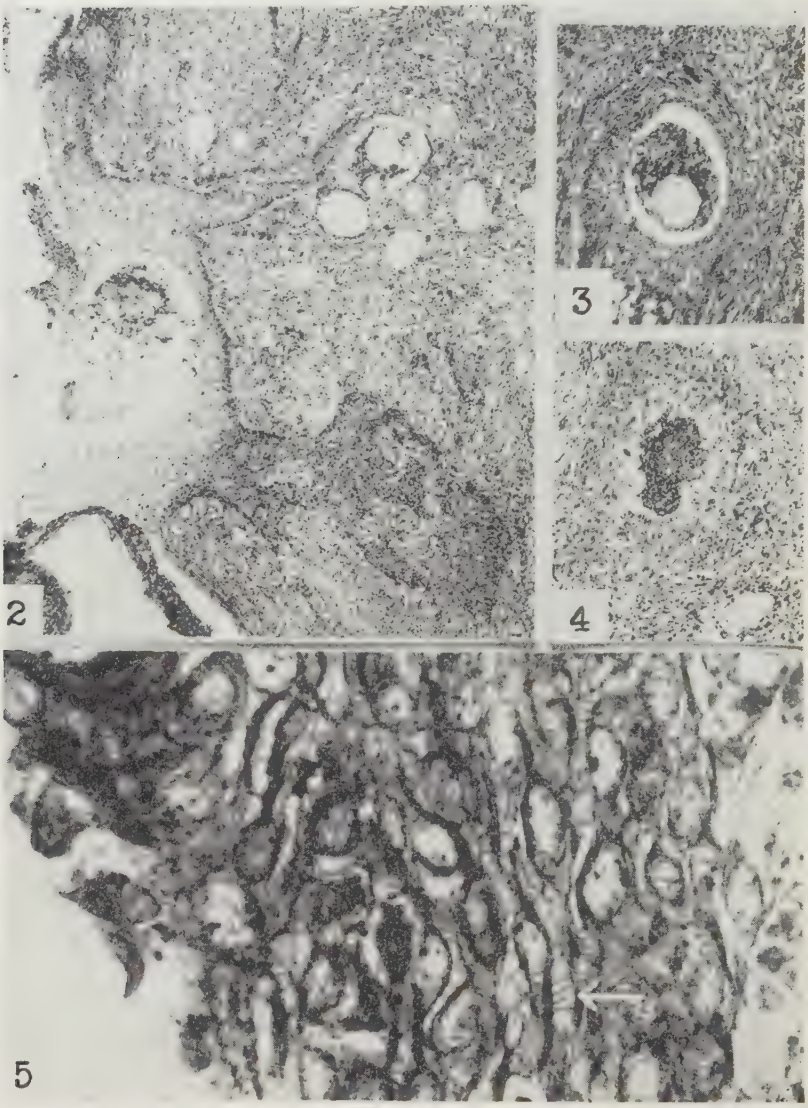

FIG. 2. (× 50) Pellet space partly lined by stratified squamous epithelium and connected with a Hassall's body. Fourteen days after implantation.

FIG. 3. (× 50) Hyperplastic Hassall's body showing beginning cyst formation at 14 days in an area devoid of small thymic lymphocytes.

FIG. 4. (× 120) Hyperplastic Hassall's body with hazy margin at seven days.

FIG. 5. (× 850) Intercellular bridges in the epithelium lining a pellet space.

cyst formation, not dissimilar to those reported here, in the thymus of rats injected with female hormones. Some of the cancerogens are estrogenic, but Gardner reports[7] that methylcholanthrene in 10 mg

[7] Gardner, W. U., personal communication.

amounts is not, so that in these experiments methylcholanthrene apparently exerted its effects as a growth promoter other than as an estrogen.

Summary. Methylcholanthrene pellets implanted into the thymus gland of young guinea pigs induced hyperplasia of Hassall's bodies and of the thymic reticulum near the pellet, squamous epithelialization of the pellet space, small epithelial cysts, and degeneration of the small thymocytes. These new epithelia show intercellular bridges and keratohyaline formation, and since they arise from Hassall's bodies they show the epithelial nature of the latter.

13466

Influence of Sex on Resistance to Intraperitoneal Inoculation of Sarcoma in Mice.

LUDWIK GROSS. (Introduced by L. H. Schmidt.)

From the Institute for Medical Research, Christ Hospital, Cincinnati, Ohio.

Previous studies performed in this laboratory have shown clearly that adult male mice are more susceptible than females to intradermal inoculation of sarcoma S 37, as evidenced by the greater incidence of takes and the lower incidence of spontaneous regression of the cutaneous tumors produced in males.[1]

Experiments reported in this paper were carried out to determine whether this difference in resistance between males and females is demonstrable only following intradermal inoculation of sarcoma or whether a similar sex difference can be demonstrated following implantation of this tumor by another route such as the intraperitoneal route of inoculation.

Experimental. Groups of male and female mice were inoculated intraperitoneally with equal doses of cell suspensions of sarcoma S 37. Tumor-cell suspensions, varying in concentration from 1 to 20%, were prepared in the usual manner[1] and injected with a tuberculin syringe and a 27-gauge needle. Sexually mature male and virgin female mice of the albino E. S. strain[2] were used throughout this study. These animals were 2 to 3 months old and weighed 18 to 24 g.

Eighteen of the 209 males and 51 of the 224 females did not react to the inoculation. The remaining 191 males and 173 females de-

1 GROSS, L., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 273.

2 GROSS, L., *Cancer Research*, 1941, **1**, 880.

veloped tumors, which could be detected in the peritoneal cavity by palpation within 6 to 22 days after intraperitoneal inoculation. These tumors grew progressively and were uniformly fatal within 10 to 53 days following inoculation, the average survival time being 17.4 and 19.5 days for males and females respectively. At autopsy single or multiple sarcomatous tumors were found in the peritoneal cavity; in many cases hemorrhagic ascites were also present. In addition, many animals developed tumors in the abdominal wall at the point where the inoculating needle pierced the skin, muscles and peritoneum.

As shown in Table I, substantially more males than females developed fatal tumors following intraperitoneal inoculation of small doses of sarcoma. Only 12 of 101 males did not develop tumors as compared with 45 of 110 females. There was only one experiment in which more females developed tumors than males, and the number of animals in this experiment was very small.

This difference in resistance between males and females was discernible, however, only when the more dilute suspensions of tumor cells were inoculated. As shown in Table II, larger doses of sarcoma produced fatal tumors in practically all animals of both sexes—only 6 of 108 males and 6 of 114 females failing to react. The slight

TABLE I.
Intraperitoneal Inoculations of Small Doses of Sarcoma S 37.

Exp. No.	Tumor suspension		Sex	No. of mice inoculated	No. of mice died with tumors	% mortality
	% conc.	cc inj.				
1	1	.03	M	18	15	83
			F	21	11	52
2	1	.10	M	20	17	85
			F	21	7	33
3	1	.30	M	17	13	76
			F	19	10	53
4	5	.03	M	5	4	80
			F	5	5	100
5	5	.03	M	20	19	95
			F	21	12	57
6	10	.03	M	5	5	100
			F	5	3	60
7	15	.03	M	16	16	100
			F	18	17	94
Summary Exp. 1-7						
			M	101	89	88
			F	110	65	59

TABLE II.
Intraperitoneal Inoculations of Medium and Large Doses of Sarcoma S 37.

Exp. No.	Tumor suspension		Sex	No. of mice inoculated	No. of mice died with tumors	% mortality
	% conc.	cc inj.				
8	20	.03	M	10	10	100
			F	10	10	100
9	20	.03	M	10	8	80
			F	10	10	100
10	20	.03	M	6	6	100
			F	6	3	50
11	20	.05	M	10	10	100
			F	10	10	100
12	20	.06	M	15	15	100
			F	15	15	100
13	20	.10	M	5	5	100
			F	5	5	100
14	20	.15	M	17	14	82
			F	18	17	94
15	20	.20	M	15	15	100
			F	14	14	100
16	20	.20	M	20	19	95
			F	26	24	92
Summary Exp. 8-16			M	108	102	94
			F	114	108	95

differences in the reactions of males and females in experiments 9, 10, 14 and 16 are probably without significance.

In the 2 series of experiments just mentioned, tumors failed to develop in 18 males and 51 females—at least no tumors could be detected in these mice by careful palpation. All 18 of the males and 29 of the females were sacrificed 48 to 56 days after inoculation; no tumors were found at autopsy of these animals. The remaining 22 females were observed for 2½ months after inoculation, then were reinoculated intradermally with 0.03 cc of a 20% sarcoma cell suspension; as controls, 20 normal female mice were inoculated simultaneously with the same tumor dose. Nineteen of the 22 reinoculated females and 18 of the 20 control animals developed sarcomas. This is an additional indication that the previous, intraperitoneal inoculation was unsuccessful, since it is known that mice that have recovered spontaneously from a successful inoculation of tumor usually resist reinoculation with the same neoplasm; this is especially true of females.[2]

Discussion. According to experiments reported in this paper, the incidence of takes following *intraperitoneal* inoculation of small doses of sarcoma S 37 was substantially higher in male than in female mice; this difference in the resistance of males and females was abolished entirely by inoculation of larger doses of this tumor. These findings are essentially identical with our previous observations[1] on the incidence of takes following *intradermal* inoculation of the same sarcoma.

It is interesting to note that Bittner[3] and Strong and his coworkers[4] have observed a similar difference in the incidence of takes in male and female mice inoculated subcutaneously with transplantable carcinomas. In their experiments no attention was paid to the importance of careful dosage of the tumors. The results that we have obtained in both intradermal and intraperitoneal inoculations of sarcoma S 37 show that careful dosage of the tumor-cell suspension is most important for demonstrating at will the influence of sex on resistance to implantation of a neoplasm. Without careful dosage of the tumor, a difference in the incidence of takes in males and females may occur only accidentally. The trocar method of grafting tumor tissue, which has been used by most investigators in the study of transplantable neoplasms, makes it almost impossible to control the tumor dosage carefully. This may explain why no convincing evidence of a sex difference in the incidence of takes was obtained previously.

As shown in the present study, tumors produced by intraperitoneal inoculation of sarcoma S 37 were uniformly fatal. Spontaneous regression of such tumors was not observed either in males or in females. This result is in marked contrast to our previous findings concerning the spontaneous regression of tumors produced by *intradermal* inoculation of the same sarcoma.[1] It should be pointed out that the most remarkable difference between the reaction of males and females to intradermal inoculation of sarcoma was the low incidence of spontaneous recovery from tumors resulting in males as compared with that in females. This sex difference in the incidence of spontaneous regression of tumor seems to be more independent of dosage than the difference in the incidence of takes.[2]

The difference in resistance of males and females to tumor implantation does not seem to be limited to mice, for Greene[5] has

[3] Bittner, J. J., *Am. J. Cancer*, 1932, **16**, 322.

[4] Strong, L. C., Hill, R. T., Pfeiffer, C. A., and Gardner, W. U., *Genetics*, 1938, **23**, 585.

[5] Greene, H. S. N., *J. Exp. Med.*, 1940, **71**, 305.

observed that twice as many takes occurred in male as in female rabbits following transplantation of a spontaneous tumor into the anterior chamber of the eye; moreover, Greene found that all grafts which progressed to the extent of corneal invasion occurred in males.

Summary and Conclusion. Following intraperitoneal inoculation of small doses of sarcoma S 37, 89 of 101 males (88%) developed tumors, as compared with 65 of 110 females (59%). This difference in resistance between males and females was completely abolished by larger doses of sarcoma, which produced tumors in 102 of 108 males (94%) and in 108 of 114 females (95%).

Tumors produced by intraperitoneal inoculation of sarcoma were uniformly fatal and in no instance was spontaneous regression of such a tumor observed.

Comparison of these results with those obtained by intradermal inoculations of sarcoma S 37[1] suggests that careful dosage of the tumor-cell suspension and the route of inoculation are most important in demonstrating the influence of sex on resistance to transplantable neoplasms. Neither of these factors has been stressed sufficiently, heretofore, in studies on the evolution of implanted tumors in different sexes.

13467 P

Experimental Atherosclerosis and Soya Lecithin.*

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The well established lipotropic property of lecithin in the prevention of fatty livers and the partial protection apparently afforded by soy bean flour against cholesterol-induced atherosclerosis in rabbits,[1] suggested the investigation of the effect of soya lecithin on experimental atherosclerosis. Accordingly 23 young adult chinchilla rabbits were divided into 3 groups. All were fed 150 mg of cholesterol daily in oil added to a basic diet consisting of white flour, alfalfa, linseed meal, carrots and salt mixture.[1] Group A received nothing more. Rabbits of Groups B and C received 5 g and 1 g respectively in the diet daily of crude soya lecithin (approxi-

* This work has been aided by a grant from the American Lecithin Company, Inc.

mate composition: lecithin 20%, cephalin 20%, oil 30%, phytosterols 2%, inositol and allied compounds 15%, carbohydrates 10%). The oil content of the several diets was adjusted to equality. The rabbits were killed after 4 months of feeding and the aortas and viscera examined, both grossly and histologically.[1]

Table I indicates the findings. Seven of the 8 animals receiving cholesterol alone developed atherosclerosis of the aorta (for the most part of moderate degree). Only 2 of the 7 in Group B that received the addition of 5 g of soya lecithin daily developed lesions (of minimal degree), and 2 of the 8 in Group C that consumed 1 g of lecithin. The average level of blood cholesterol of the rabbits, in Group A, attained 430 mg (± 150) per 100 cc, in Group B (5 g lecithin) 210 mg (± 75), and in Group C (1 g lecithin) 300 mg (± 100). The livers of all the rabbits were normal grossly and histologically.

How much of this protective effect of lecithin on cholesterol deposition is due to choline is problematical. Choline has been found by Steiner,[2] Baumann and Rusch,[3] and Himsworth[4] to have no effect on the hypercholesterolemia of cholesterol-fed animals. Baumann and Rusch, and Himsworth reported also no effect on cholesterol deposition in the aorta, but Steiner observed a delay in the production of atherosclerosis. A 4th group of rabbits received 195 mg of choline chloride per rabbit per day (equivalent to the choline content of 5 g of crude soya lecithin). The incidence of atherosclerosis was sharply less than in the animals receiving cholesterol alone, but only a little greater in degree than in the groups fed lecithin. However, hypercholesterolemia in this group [340 mg (±160) per 100 cc] was more severe than in the animals receiving 5 g of lecithin daily. It

TABLE I.
Effect of Soya Lecithin on Cholesterol Sclerosis.

Diet	Rabbits			Degree of sclerosis of aorta	
	No. used	No. sclerotic	Percentage sclerotic	+	++
A. Basic	8	7	88	2	5
B. Lecithin 5 g	7	2	28	2	0
C. " 1 "	8	2	25	1	1
D. Choline	8	3	38	1	2

\+ No lesion visible grossly, or doubtful, but one was evident microscopically.
++ Sclerosis evident grossly as one to several atheromatous plaques.

[1] Meeker, D. R., and Kesten, H. D., *Arch. Path.*, 1941, **31**, 147.

[2] Steiner, A., PROC. SOC. EXP. BIOL. AND MED., 1931, **38**, 231.

[3] Baumann, C. A., and Rusch, H. P., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 647.

[4] Himsworth, H. P., *Acta Med. Scand. Suppl.*, 1938, **90**, 158.

seems likely that, with this high blood cholesterol, the animals fed choline would shortly have developed more extensive atherosclerosis. Further work is needed to clarify this point.

Summary. The feeding of soya lecithin to rabbits receiving cholesterol restricts hypercholesterolemia and diminishes the incidence of experimental arteriosclerosis.

13468 P

Nerve Concussion.

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Recent observations[1] indicate that typical cerebral concussion is best reproduced in unconscious animals by subjecting the head to a sufficiently high rate of change in velocity. The response is an immediate muscle "start reflex" followed at once by relaxation and abolition of reflexes (but with continuing discharge from reflex centers) lasting up to 60 seconds; recovery (or death) may occur within a period of 5 minutes.

In the present experiments a short segment of the sciatic nerve of the green frog is compressed by a blast from an air pistol. The segment exposed to the blast lies on a solid foundation at the bottom of a pit 3.6 mm wide and about 4 mm deep into which the air is shot. "Maximal" induction shocks delivered to the nerve centrad to the compressed segment test the degree of the resulting conduction block, either in terms of contraction height, when the muscle (gastrocnemius) remains attached, or in terms of area of the monophasic action potential when the nerve responses are recorded with the cathode ray oscillograph. "Maximal" shocks delivered to the nerve distad to the compressed locus control the condition of the nerve beyond the pit, and of the muscle.

A blast that blocks, either partially or completely, may elicit a maximal twitch of the muscle due to the mechanical stimulation of the nerve. Testing shocks applied immediately thereafter reveal a reduction, it may be to zero, in the height of the contraction or of the spike. The test contractions or spikes then almost immediately

1 Denny-Brown, D., and Russell, W. R., *Brain*, 1941, **64**, 93.

increase in height and this progresses steadily through periods that have lasted up to 10 minutes, but usually not longer than 3 to 5 minutes. We have not yet found a blow that permits of complete recovery from a complete block. With the muscle as the index, the best recovery thus far obtained has been to 80% of the original height from a reduction by the blast to 45% of the original height. A maximum *A* action potential, though, has been reduced by the blast to 24% of its original area and at maximum recovery, attained in 4.3 minutes, had 89% of the original. Failure to obtain complete recovery from complete block possibly is referable to the manner of exposure of the nerve to the blast: it is driven against the foundation it rests on and the fibers therefore are not all compressed equally; possibly it is referable also to a difference in the susceptibility to blast of fibers of different sizes. The gradualness of the recovery is the expression of differences in the rate of recovery of the individual component fibers from block.

Compression block must be, in effect, a stretch block, since localized compression deforms that locus, narrowing and therefore stretching it, whereas uniform compression does not annul excitability.[2] Denny-Brown and Russell are not definite regarding "the mechanism by which the neurones are damaged in acceleration concussion," though they do say that "the demonstration of a threshold for the necessary acceleration indicates that a fixed uniform physical change" is concerned. Again, they attribute the paralytic phenomena to "direct generalized physical injury to neurones" which causes immediate, but reversible, loss of function, and state that the hemorrhagic lesions which occur in very severe injuries indicate distortion or stress, but not necessarily identical with that which causes concussion, and that petechial hemorrhages, when they occur, probably are produced by "direct squashing or stretching."

The fact that the events of nerve and cerebral concussion run approximately the same time course suggests that the mechanism and the structures involved are the same in both. But how, then, can the combination of continued activity of nerve centers with concomitant paralysis of reflexes be accounted for? It might be due (a) to a fundamental difference in the reaction of cell and fiber to stretch; or (b) to a more protected position of the centers or to differences in the lengths exposed to stretch, fibers being long and cells short, with the additional proviso that cells respond with repetitive discharges to stretches of less than blocking intensity; or (c) to

[2] Cattell, McK., and Edwards, D. J., *Science*, 1930, **71**, 17.

an association with neurone block of repetitive discharges from the injured, the blocked, locus.

In favor of this last possibility is the observation we have made twice now, that fibers that have been blasted continue to develop spikes after the one initiated by the blast. But if this happens in experimental cerebral concussion, one would expect the initiating "start reflex" to be followed by some evidences of motor activity. As a matter of fact, Denny-Brown and Russell state that the start reflex is followed by a delayed spasm and then by the loss of postural tone. Concussion would be accounted for, on this basis, by stretch blocks of neurones plus excitation at the distorted loci, when there are evidences of excitation.

Summary. An adequate blast from an air pistol applied to a short segment of nerve causes the fibers to discharge once but immediately blocks the transmission of impulses. Fibers thus blocked may initiate repetitive spikes. Within the course of 3 to 5 minutes, as a rule, conductivity returns spontaneously in all fibers that have not been irreparably damaged. The possible relation of these results to cerebral concussion is discussed.

13469 P

Carbohydrate Metabolism and Staphylococcus Infection in Rabbits.

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A previous communication from these laboratories[1] showed that spreading necrotising staphylococcal skin infections and subcutaneous injections of necrosing doses of staphylococcus toxin produced temporary but definite glucose intolerance in rabbits. It was thought that it would be of interest to observe the effect of repeated subcutaneous injections of *Staphylococcus aureus* and of staphylococcus toxin. The following communication gives the results of some preliminary experiments of this type.

Experimental Procedure. The methods of analysis and injection were the same as described previously. Twelve rabbits were used.

1 Jackson, S. H., Nicholson, T. F., and Holman, W. L., *J. Path. and Bact.*, 1940, **50**, 1.

Six were twice injected intradermally with *Staphylococcus aureus,* an interval of 3 weeks elapsing between each injection. The infection following each injection was the spreading necrotic type of lesion described by Jackson, *et al.*[1] The other 6 rabbits were twice injected intradermally, first with 2 ml of a 1:20 dilution of staphylococcus toxin and then, after an interval of 3 weeks with the same amount of undiluted toxin.

Glucose tolerance curves were performed 4 days after each injection and then at monthly intervals for 5 months. Before the end of the sixth month all the rabbits succumbed to an epidemic of snuffles. There were, however, no signs of snuffles until 2 weeks after the blood was taken for the last tolerance curve. The urine of all rabbits was examined for sugar periodically.

Results. All the animals developed slight glycosuria in the 24-hr specimens in from one to 2 months after the second treatment with *Staphylococcus aureus* or of staphylococcus toxin. This glycosuria increased in severity over a period of from 4 to 6 weeks until it was quite marked with occasional traces of glucose in the fasting urine.

The effects of *Staphylococcus aureus* infection and of Staphylococcus toxin on the glucose tolerance curves of rabbits.

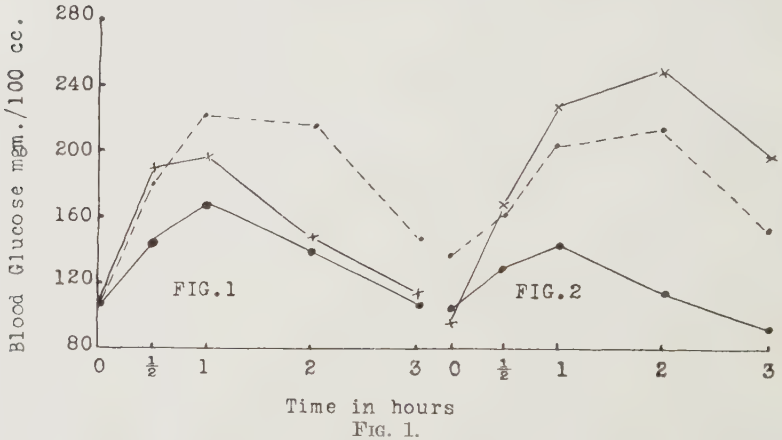

FIG. 1.

Composite glucose tolerance curves showing the effect of subcutaneous infection with *Staphylococcus aureus.* 1st infection April 10th, 1940, 2nd infection May 6th, 1940.

● ——— ● April 9th, 1940.
× ——— × April 13th, 1940.
● - - - - - - - ● September 9th, 1940.

FIG. 2.

Composite glucose tolerance curves showing the effect of intradermal injections of staphylococcus toxin. 1st injection (1:20 dilution) April 10th, 1940, 2nd injection (undiluted) May 6th, 1940.

● ——— ● April 9th, 1940.
× ——— × April 13th, 1940.
● - - - - - - - ● September 7th, 1940.

The effects on glucose tolerance curve are shown in Fig. 1 (staphylococcus infection) and 2 (intradermal staphylococcus toxin). In both cases there was evidence of a decrease in carbohydrate tolerance with a marked increase in the peak of the curve and a delayed return to fasting levels. This was quite marked following the second injection of toxin, the curve here presenting many points of similarity to that seen in mild diabetes.

Discussion. It is evident that repeated injections of *Staphylococcus aureus* or staphylococcus toxin accentuates and makes permanent the decrease in glucose tolerance which had been observed following a single lot of injections. The exact cause of this decreased tolerance has not been determined. Some preliminary experiments on rats, however, have shown that repeated injections of staphylococcus toxin may lower the insulin content of the pancreas.[2]

The effect of a greater number of injections of both organisms and toxin are being studied to determine whether or not a more severe glucose intolerance, simulating diabetes, may be produced.

Summary. Two massive doses of *Staphylococcus aureus* or staphylococcus toxin produced a definite and permanent decrease in carbohydrate tolerance, with glucose tolerance curves similar to those seen in mild diabetes.

13470

Electrophoretic Identification of Antibody to Tuberculin Protein.*

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It has been shown[1, 2] that rabbits can be readily sensitized to tuberculin protein by repeated injections of the antigenic form of the protein. The sensitization can be demonstrated by means of the precipitin test,[1] the Arthus reaction,[2] anaphylaxis and the uterine strip contraction.[3] Obviously antibodies to the tuberculin protein

2 Nicholson, T. F., and Haist, R. E., unpublished results of work done in the Departments of Pathological Chemistry and Physiological Hygiene, University of Toronto.

* Aided by a grant from the Committee on Medical Research of the National Tuberculosis Association.

1 Seibert, F. B., *Am. Rev. Tuberc.*, 1930, **21**, 370.

2 Seibert, F. B., *J. Infect. Dis.*, 1932, **51**, 383.

3 Lewis, J. H., and Seibert, F. B., *J. Immunol.*, 1931, **20**, 201.

are present in the blood stream, as is the case with other protein sensitizations.

Antibodies to the pneumococcus[4–7] and to tetanus antitoxin[8] have been identified in the respective antisera not only by the well-known immunological technics but also recently by means of the Tiselius electrophoresis apparatus.[9] They have been shown to exist as components with mobilities similar to or close to that of normal γ-globulin.

It seemed worth while to attempt the identification of the antibody to tuberculin protein by this method. The following experiments approached the problem in two different ways. (1) Changes in the blood sera of rabbits were noted during sensitization with the tuberculin protein. Table I shows that in seven such sensitized rabbits the percentage of the α-globulin fraction was raised, while the albumin decreased. The ranges and average percentages of the serum proteins of 12 normal rabbits are given for comparison. (2) Immune

TABLE I.

Serum	Total conc. %	% of total as Albumin	Globulins α	Globulins β	Globulins γ	% α-globulin removed
Avg of 12 normals	5.4	76.0	1.1	10.8	12.2	
Range	(4.5-6.2)	(72.2-81.5)	(0-2.5)	(8.4-12.9)	(9.4-14.3)	
No. 7	5.4	61.0	6.7	11.6	20.7	
No. 82	5.8	67.7	8.6	12.2	11.5	
No. 84	7.2	70.7	6.9	10.4	12.0	
No. 2083	6.5	58.0	7.9	14.1	20.1	
No. 140	6.5	70.6	5.2	10.2	13.9	
No. 140 specific precipitate removed	6.6	71.5	3.6	10.5	14.5	30.7
No. 3	5.8	59.8	8.5	14.6	17.1	
No. 3 " "	6.2	60.4	7.2	14.9	17.6	15.3
No. 196	5.0	64.0	8.7	12.6	14.8	
No. 196 " "	4.5	63.5	7.8	13.9	14.4	10.3

All electrophoretic analyses were made on sera diluted 1:4 in phosphate buffer, pH 7.7, $\mu = 0.1$, with a potential gradient of 6.6 to 7.1 volts/cm. Each figure represents the average concentration calculated from four Svensson-Philpot curves. All antisera gave precipitin titers of 1:100,000 or 1:200,000 with the specific antigen.

[4] Tiselius, A., and Kabat, Elven A., *J. Exp. Med.*, 1939, **69**, 119.

[5] Blix, G., *Z. ges. exp. Med.*, 1939, **105**, 595.

[6] Moore, D. H., van der Scheer, J., and Wyckoff, R. W., *J. Immunol.*, 1940, **38**, 221.

[7] van der Scheer, J., Lagsdin, J. B., and Wyckoff, R. W., *J. Immunol.*, 1941, **41**, 209.

[8] van der Scheer, J., and Wyckoff, R. W., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 427.

[9] Tiselius, A., *Trans. Faraday Soc.*, 1937, **33**, 524.

sera (Nos. 140, 3, and 196) which showed high globulin percentages were precipitated with the homologous antigens at the equivalence point, the precipitates centrifuged off and then the remaining sera were studied in electrophoresis under conditions identical to those employed with the original serum, in order to determine in what fraction a loss might have occurred. The table shows that the α-globulin fraction was partially decreased in these experiments.

Therefore, it would appear that the antibody to the tuberculin protein may exist in immune sera as a component with a mobility similar to or very close to that of α-globulin, which in the normal rabbit is extremely small in amount.

It is probable that this antibody is not important as a defense mechanism against the disease tuberculosis since it was shown earlier[10] that guinea pigs rendered highly sensitive to the tuberculin protein so that they exhibited typical Arthus reactions and high precipitin titers, were not protected against infection. They were, in fact, more susceptible. Further attempts are being made to detect other antibodies with possibly greater significance in immunity.

An unknown component, designated as χ-component, appeared in

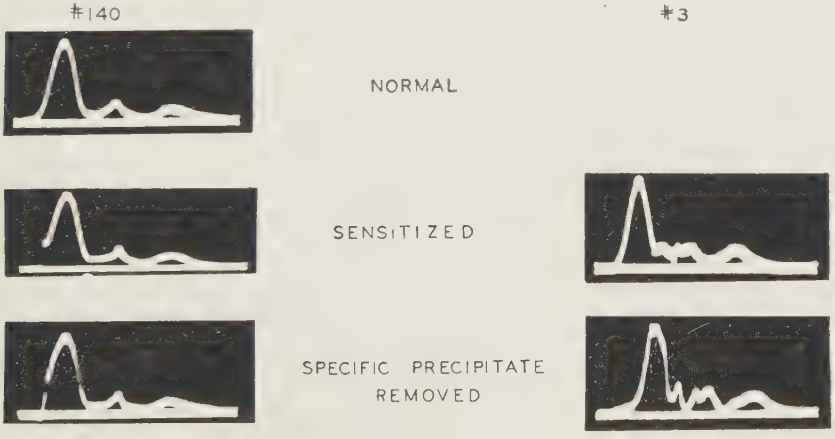

FIG. 1.

Electrophoretic (Svensson-Philpot) diagrams of 2 immune sera; descending side. No. 140 shows the picture in the normal serum where practically no α-globulin is visible; serum after sensitization, where the increase in α-globulin is seen as a definite rise from the base-line and the fast component appears as a break in the leading side of the albumin curve; serum after specific precipitate is removed, showing the return of the α-curve to the base-line. Serum No. 3 shows a more significant rise in the α-globulin, but the decrease of 15% in α-globulin when the specific precipitate is removed, is better noted by accurate measurement than by observation.

10 Seibert, F. B., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1274.

most sera during sensitization. It had a mobility slightly greater than albumin and can be seen in the diagrams for rabbit No. 140. In another communication[11] it has been suggested that this may be due to the presence of tuberculin protein in the serum.

An increase in the α-globulin component occurred also in a rabbit sensitized in a similar manner with crystalline ovalbumin, and may, therefore, possibly be a general phenomenon in protein sensitization.

Conclusion. Antibody to tuberculin protein has been identified in serum as a component with an electrophoretic mobility close to α-globulin. This component increases in sera following sensitization to the protein, and is removed in part with the specific precipitate produced by adding antigen to the immune serum.

13471

Urinary Excretion of Pantothenic Acid by Normal Individuals.

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Excretion studies have frequently been employed in assessing the dietary requirement for certain nutritive essentials. It is axiomatic that individuals consuming diets deficient in a particular nutritive essential will excrete less of the material in question than those subsisting on an adequate or optimum regimen. The establishment of normal excretion values is, therefore, a requisite for the application of such a method in evaluating the adequacy of diets.

Pantothenic acid is a dietary essential for a variety of species. Evidence has been presented by Spies, *et al.*,[1] that it is essential in human nutrition. Pelczar and Porter,[2] using a method based on the essential nature of pantothenic acid for *Proteus morganii,* found that the pantothenic acid content of 24-hour urine specimens from 9 persons ranged from 1.46 mg to 6.79 mg and averaged 3.81 mg. Pearson,[3] using the method of Pennington, Snell, and Williams,[4]

11 Seibert, F. B., and Nelson, J. W., *J. Biol. Chem.*, in press.

1 Spies, T. D., Stanbery, S. R., Williams, R. J., Jukes, T. H., and Babcock, S. H., *J. Am. Med. Assn.*, 1940, **115**, 523.

2 Pelczar, M. J., and Porter, J. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 3.

3 Pearson, P. B., *Am. J. Physiol.*, 1941, **135**, 69.

4 Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **135**, 213.

which employs *Lactobacillus casei* as the test organism, reported that the amount of pantothenic acid excreted during a 48-hour period by each of 3 individuals was 6.32, 6.60, and 6.23 mg. The daily excretion of pantothenic acid, therefore, is in agreement with that reported by Pelczar and Porter. In this paper data are reported on the pantothenic acid content of 57 24-hour urine specimens from 29 normal individuals (medical students).

Two 24-hour specimens were collected from each subject approximately one week apart; toluene was used as a preservative. The specimens were assayed according to the method of Pennington, Snell, and Williams.[4] An acid-treated sample of urine was incorporated in the basal medium as suggested by these workers. Under such conditions recoveries of pantothenic acid, when added to urine, were quantitative. Titration of the lactic acid produced after a growth period of approximately 72 hours was employed as a measure of the response to pantothenic acid. The results are expressed in terms of milligrams of calcium pantothenate excreted per day.

A frequency distribution of the data obtained is presented in Table I. In 72% of the cases studied the daily excretion fell between 2.50 and 4.00 mg per day. The mean daily excretion of pantothenic acid was 3.42 mg (maximum 5.54 mg; minimum 1.10 mg).

TABLE I.
Daily Excretion of Pantothenic Acid.

Range mg per day	No. of samples
— -1.99	2
2.00-2.49	2
2.50-2.99	13
3.00-3.49	16
3.50-3.99	12
4.00-4.49	5
4.50-4.99	3
5.00-5.49	3
5.50- —	1
Total	57

13472 P

Effect of Biotin on Certain Physiological Functions.

JOHN L. SCHMIDT AND MAURICE LANDY. (Introduced by W. E. Hambourger.)

From the Research Laboratories of the S. M. A. Corporation, Chagrin Falls, Ohio.

Biotin, the factor curative of egg white injury[1] and a growth-essential for many bacterial species,[2, 3, 4] is now considered an important component of the vitamin B-complex. Up to the present time, a study of the immediate effect of biotin on physiological functions has not been undertaken. The experiments described here demonstrate the effect of biotin on blood pressure, respiration, frog heart, excised uterus and intestinal strip. Since crystalline biotin is available only in limited quantity and because of its high degree of biological activity (1 μg equals 27 vitamin H rat curative units[1]), relatively small amounts were used in carrying out this study.

Because of its low solubility in water, crystalline biotin methyl ester was hydrolyzed and the acid converted into the more soluble sodium salt. To 5 mg of the methyl ester was added 0.775 mg NaOH, after which dilution was made to 10 cc with distilled water. This mixture was heated in a pyrex tube over a free flame until solution took place (about 2 minutes).

Two small cats (2 kg each) were used for studying blood pressure and respiration. The blood pressure was recorded with a mercury manometer connected to a carotid artery, and respiration was recorded with a Marey tambour attached to a by-pass in the tracheal cannula. Rapid intravenous injection of 500 μg of biotin produced no significant change in blood pressure, heart rate and respiration (Fig. 1).

Smooth-muscle studies were made in a 20 cc excised organ bath of all-glass construction. Eight strips of guinea pig uterus, one strip of rabbit uterus and one segment of rabbit intestine were studied suspended in oxygenated Sollmann-Rademaeker's solution at 38.5°C. Concentrations of biotin up to 1 part in 40,000 did not affect the normal rhythmic contractions of these organs (Figs. 2 and 3).

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[1] György, P., Rose, C. S., Hofmann, K., Melville, D. B., and du Vigneaud, V., *Science*, 1940, **92**, 609.

[2] Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1939, **61**, 3594.

[3] West, P. M., and Wilson, P. W., *Enzymologia*, 1940, **8**, 152.

[4] Porter, J. R., and Pelczar, M. J., *J. Bact.*, 1941, **41**, 173.

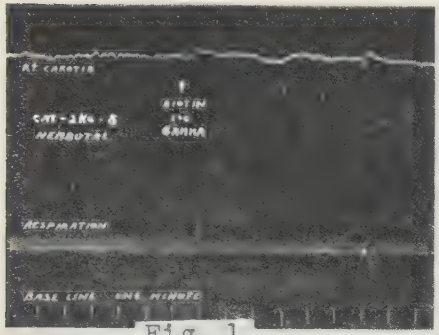

Fig. I

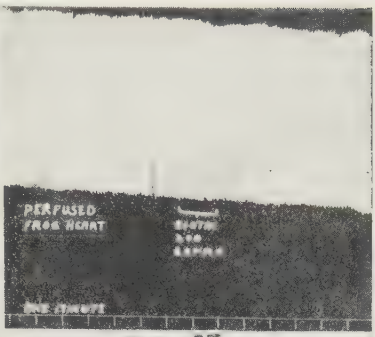

Fig. IV

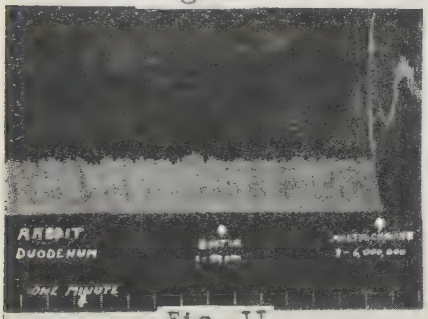

Fig. II

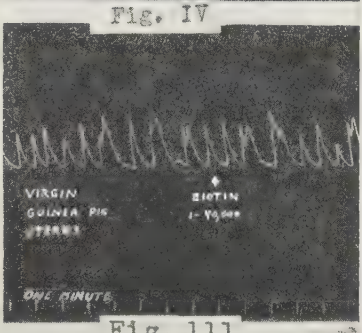

Fig. III

The effect of biotin on the frog heart was studied by perfusing this organ *in situ* through a Greene cannula tied into the vena cava. To each of 3 preparations, 200 μg (0.4 cc) of biotin was introduced through the standpipe of the cannula and washed in by the Ringer perfusate. In no case was there significant alteration in amplitude, rate or rhythm (Fig. 4).

Summary. Biotin failed to produce any changes in the following physiological functions: blood pressure, heart rate and respiration of anesthetized cats following rapid intravenous injection of 250 μg per kilo; excised guinea pig and rabbit uterus and rabbit intestine suspended in concentrations up to 1:40,000; and the frog heart perfused *in situ* with 200 μg of biotin.

13473

Adrenalectomy and the Absorption of Different Fats.*

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The effect of removal of the adrenals upon the rate of fat absorption still remains unsettled. Opposing the earlier work of Verzar and Laszt,[1, 2] Barnes, *et al.*,[3] and Barnes, Miller, and Burr[4] demonstrated that adrenalectomized rats maintained with salt absorbed corn oil (Mazola) and the methyl esters of corn oil fatty acids at the same rate as normal unoperated controls. Recently, Bavetta, *et al.*,[5] have shown that hydrogenated vegetable oil is absorbed more slowly by adrenalectomized rats and that this lowered absorption rate could be returned to normal by the administration of cortin. One outstanding difference in procedure employed by the 3 groups of investigators mentioned above is in the type of fat used in testing absorption rate. Verzar and his coworkers have used olive oil in most of their studies, Bavetta, *et al.*, employed hydrogenated vegetable oil, and workers from this laboratory have used corn oil. The present investigation was undertaken in order to determine whether or not the type of fat fed had any influence on the differences in experimental results that have been mentioned.

Procedure and Results. Adult albino rats were bilaterally adrenalectomized, and together with a group of unoperated control rats were fed a stock diet and given 0.6% sodium chloride and 0.2% sodium citrate *ad libitum* for 4 days. On the fourth day after operation, food was removed from all cages. Approximately 20 hours after removal of the food the rats were fed by stomach tube 0.5 cc of the test fat per square decimeter body surface. Eight hours following this, the rats were killed by etherization and their entire in-

* Aided by a grant from the Graduate School of the University of Minnesota. Assistance in preparation of the paper was furnished by the personnel of Works Project Administration, Official Project No. 165-1-71-124, Sub-project 325 and Sub-project 331.

1 Verzar, F., and Laszt, L., *Biochem. Z.*, 1935, **276**, 11.

2 Verzar, F., and Laszt, L., *Biochem. Z.*, 1935, **278**, 396.

3 Barnes, R. H., Wick, A. N., Miller, E. S., and MacKay, E. M., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 651.

4 Barnes, R. H., Miller, E. S., and Burr, G. O., *J. Biol. Chem.*, 1941, **140**, 241.

5 Bavetta, L., Hallman, L., Deuel, H. J., Jr., and Greeley, P. O., *Am. J. Physiol.*, 1941, **134**, 619.

testinal tracts washed out with water and alcohol in the manner previously described.[3] The intestinal washings were acidified and extracted with petroleum ether. After weighing the fat extracts, they were dissolved in methyl alcohol and titrated with a 0.1 *N* KOH solution in methyl alcohol, using phenolphthalein as an indicator.

Four different fats were employed in this study. These were corn oil (Mazola), olive oil, hydrogenated vegetable oil (Crisco), and mutton tallow (melting point 43°-45°C). The hydrogenated oil was fed both in its original form and as an emulsion made of 9 cc of the fat with 1 cc of skim milk in the manner apparently used by Bavetta, *et al.*,[5] in their studies.

The results, which are summarized in Table I, show no significant effect of adrenalectomy upon the rate of fat absorption for 3 of the fats, namely, corn oil, olive oil, and mutton tallow. In view of the variability as indicated by the calculation of standard deviation, it would also appear likely that the rate of absorption of the unaltered hydrogenated oil is not significantly altered by adrenalectomy. The one experiment in which the rate of absorption seems definitely lowered in the adrenalectomized rats is the one in which an emulsion of hydrogenated oil in skim milk was fed. This confirms the observation of Bavetta, *et al.*,[5] who apparently employed this fat emulsion in their studies, but peculiarly enough it is the only one of the

TABLE I.
Absorption of Different Fats During an 8-hour Period by Adrenalectomized and Unoperated Control Rats.*

Fat Fed	Treatment	No. of rats	Avg body surface cm^2 [7]	Avg fat absorbed per cm^2 B.S. mg†	Avg free fatty acids in extract mg†
Corn oil	Control	6	453	3.39±0.46	205±95
" "	Adrenalectomized	6	449	2.91±0.33	340±43
Olive "	Control	6	455	3.24±0.33	301±140
" "	Adrenalectomized	5	453	3.23±0.84	329±264
Hydrogenated oil	Control	6	457	3.18±0.26	134±48
" "	Adrenalectomized	5	458	2.39±0.79	286±78
Emulsified hydrogenated oil	Control	6	461	2.73±0.28	187±59
" "	Adrenalectomized	5	448	1.72±0.35	306±200
Mutton tallow‡	Control	5	445	1.83±0.53	141±45
" "	Adrenalectomized	5	455	1.98±0.32	214±27

*All rats except those fed mutton tallow were adult females. Rats receiving mutton tallow were adult males.

†Including the standard deviation.

‡Melting point 43°-45°C.

[7] Carman, G. G., and Mitchell, H. H., *Am. J. Physiol.*, 1926, **76**, 380.

5 different fat preparations shown in Table I, which has a definitely lowered rate of absorption.

The extent of variability in free acids, as indicated by the standard deviation calculations, is a common observation in this type of study. This variation is so large that it would be untenable to conclude from the data presented in Table I that a definite difference in free fatty acid content existed between the normal control and adrenalectomized rats. It is interesting that from the averages alone the free fatty acids of the intestinal contents are higher in all of the adrenalectomized groups than in their controls. The fact that other investigators[5, 6] have observed an accumulation of free fatty acids in the intestines of adrenalectomized rats after fat feeding lends some support to the significance of the data presented here. However, all observations previously made have referred to fatty acid accumulation in adrenalectomized rats exhibiting a lowered rate of fat absorption, while it would appear from these data that perhaps such an accumulation may take place in adrenalectomized rats that are absorbing fat at a normal rate.

It has been mentioned by other investigators[5, 6] that the characteristic milky appearance of the mesenteric lacteals in the normal rat after feeding fat is decreased or entirely absent in the adrenalectomized rat which has been fed fat. This has been interpreted as further evidence that fat absorption is impaired in the adrenalectomized animal. In the present study this same phenomenon was observed. However, it was noticed that the lessened milky appearance of the mesenteric lacteals was common to all of the adrenalectomized rats and was not restricted to just those groups in which fat absorption had been decreased.

From the above discussion it is seen that in the adrenalectomized rat the accumulation of free fatty acids in the intestine and the gross appearance of the intestine during fat absorption may differ from the normal even though the actual rate of fat absorption is unchanged. These facts, together with the previously quoted observation[4] that histochemical examination of the intestinal mucosa of adrenalectomized rats absorbing fat at a normal rate shows a definite alteration from the normal, must be taken as evidence that rate of absorption alone need not indicate changes in the fat absorbing mechanisms that have been brought about by removing the adrenals.

Summary. The rate of absorption of corn oil (Mazola), olive oil, hydrogenated vegetable oil (Crisco) and mutton tallow is not sig-

[6] Verzar, F., and McDougall, E. J., *Absorption from the Intestine*, Longmans, Green and Co., 1936.

nificantly changed from the normal by adrenalectomy. The absorption of hydrogenated vegetable oil fed as an emulsion with skim milk is significantly decreased following this operative procedure. Evidence for certain changes in fat absorbing mechanisms that are brought about by removal of the adrenals is presented, and it is pointed out that these changes need not result in an altered rate of absorption.

13474

Effect of Some Sulfonamides on Renal Secretion.*

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In studies concerning the correlation between the molecular configuration of organic compounds and the secretory power of the kidney it has been found that the functional behavior of the isolated frog kidney can be influenced in various ways by sulfanilamide and its derivatives. Among these influences is one, which recently came to our attention and which regards a striking and easily observable pH shift in the secretion from an acid towards an alkaline reaction.

Method. The kidney of frogs *(R. pipiens)* was perfused from the aorta or from both the aorta and the abdominal vein with bicarbonate-Ringer aerated by O_2-CO_2 in a mixture providing a pH of about 7.5. The perfusion pressures were 20 to 24 cm when the aorta alone was perfused and 24 and 12 cm respectively, when both vessels were perfused. 0.5 to 0.25 mg % phenol red was added. At this time of the year (October, November) the secretion appearing in the ureter cannulas under these conditions is ordinarily canary-yellow or dark orange-yellow. This indicates an acid reaction of the secretion and a marked concentration of the dye, both being due to the activity of the convoluted tubules. The secretion was taken up drop by drop on white paper and the color shade of each drop noted. This

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The sulfanilamide derivatives were generously furnished by the Calco Chemical Division of the American Cyanamid Company, the Winthrop Chemical Company, Merck & Company, Sharp & Dohme, and Abbott's Dermatological Research Laboratories (through the courtesy of Dr. G. W. Raiziss).

was necessary since the color changes in contact with air by loss of CO_2.

Results. After adding sulfanilamide to the perfusion fluid, the color of the successive drops changes from yellow over a series of orange and red shades to pink, indicating a continuous shift from an acid to an alkaline reaction. This occurs in a few minutes. The process is reversible. Omission of the drug from the perfusion fluid allows the color to change back stepwise to yellow. The entire sequence of changes can be reproduced several times with the same frog preparation. The rate and the intensity of the color changes depend upon the drug concentration. The threshold concentration was found between 5 and 10 mg %.

Several sulfanilamide derivaties have been studied. An effect resembling that of sulfanilamide is brought about by *N^4-acetylsulfanilamide* (Calco), 0.05 and 0.01%, *N^4-sulfanilylsulfanilamide* (Calco), satur. = <0.03%, *N^4-butyrylaminobenzenesulfonamide* (Sharp & Dohme), satur., *N^4-sodium p-sulfamidophenylglycine* (Abbott) = $NaOOC\text{-}CH_2 . NH . C_6H_4 . SO_2NH_2$, 0.025 to 0.1% and *$N^4$-sodium methylenesulfonate of sulfanilamide* (Abbott) = $NaSO_3 . CH_2 . NH . C_6H_4 . SO_2NH_2$, 0.05%. In contrast to the N^4-compounds, the color change fails to appear after perfusing the kidney with the N^1-compounds—*sulfapyridine* (Merck), satur. = <0.05%, *Sulfaguanidine* (Calco), 0.1%, and *Sulfadiazine* (Calco), satur. = <0.01%.† The effect of sulfanilylsulfanilamide is irreversible.

Discussion. It is evident that the capacity of the drugs to turn the reaction of the renal secretion from acid to alkali appears so far to be confined to compounds with an unsubstituted sulfonamide group. The failure of sulfadiazine to act could be due to its low solubility, but sulfapyridine, which is also badly soluble, was perfused without effect at a molarity 5 to 10 times as great as that of sulfanilamide at its threshold concentration.

This behavior of the drugs in relation to the kidney conforms with their behavior in inhibiting carbonic anhydrase as observed by Mann and Keilin.[1] According to these authors, carbonic anhydrase is inhibited by sulfonamides having an unsubstituted N^1 group and not sulfonamides having a substituted N^1 group, irrespective of substitution on the N^4 group. The activity of many other enzymes is

† The substitutes of the amido group in sulfanilamide are called N^1-compounds, the substitutes of the amino group N^4-compounds.

[1] Mann, T., and Keilin, D., *Nature*, 1940, **146**, 164.

not affected by sulfanilamide concentrations far exceeding the concentration which abolishes carbonic anhydrase activity.

It is suggestive, therefore, to interpret the pH shift in the renal secretion observed in our experiments as due to the inactivation of carbonic anhydrase. This interpretation would seem consistent with the following facts: First, Davenport[2, 3] has provided evidence that in the parietal cells of the stomach glands, which generally are looked upon as being the site of production of the gastric acid, carbonic anhydrase is present in a concentration even higher than in red cells. Accordingly, the acid secretion of the gastric mucosa is diminished by sulfanilamide. Second, Davenport and Wilhelmi[4] have shown carbonic anhydrase to be present in a significant concentration in the cortex of cat, dog and rat kidneys and Davenport[5] also has shown that carbonic anhydrase is present in the frog kidney.

It seems promising to consider the mechanism of this and other glandular pH shifts on the basis of our experiments. It has been discussed previously, whether the acid reaction of the frog's renal secretion rests upon the reabsorption of HCO_3 or upon the excretion of H. In this regard it is an important fact that, according to Montgomery and Pierce,[6] the acidification of the secretion takes place in a short segment of the distal tubules nearer to their distal than to their proximal end, whereas, according to Walker, Hudson, Findley and Richards,[7] Cl is reabsorbed along the total length of the distal tubules. This is indicative of two independent mechanisms being involved in the normal decrease of OH and of Cl inside the tubules.

This conclusion is corroborated by our further observation that sulfanilamide fails to influence the Cl shift simultaneously with the pH shift. It often happens that the concentration of Cl in the secretion rises during the course of perfusion with Ringer. However, in 15 experiments no correlation whatsoever appeared between the concentration of Cl and the reaction. If the experiment is started with perfusion with Ringer plus sulfanilamide, the Cl concentration is not found to be lower during a subsequent period of perfusion with Ringer alone, although the reaction has become acid. Likewise, if sulfanilamide is added in any one of a series of periods

2 Davenport, H. W., *J. Physiol.*, 1940, **97**, 32.

3 Davenport, H. W., *Am. J. Physiol.*, 1941, **133**, 257.

4 Davenport, H. W., and Wilhelmi, A. E., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 53.

5 Unpublished observations.

6 Montgomery, H., and Pierce, J. A., *Am. J. Physiol.*, 1937, **118**, 144.

7 Walker, A. M., Hudson, C. L., Findley, T., and Richards, A. N., *Am. J. Physiol.*, 1937, **118**, 121.

of perfusion, the sample of secretion corresponding to this period does not show an especially marked increase of Cl concentration.

For all these reasons it seems adequate to assume that the normal pH shift from an alkaline to an acid reaction is due to the localized reabsorption of HCO_3. This will bring about a pH shift from 7.4, corresponding to the normal pH of the blood and of our perfusion fluid, to pH 4.8, equal to our perfusion fluid without HCO_3 and equal to the lowest pH value of frog's urine.[6] This reabsorption can be looked upon as being causally related to the catalyzing effect of carbonic anhydrase.

Summary. Following the addition of sulfanilamide and of sulfonamides having an unsubstituted SO_2NH_2 group to the Ringer-phenol red perfusion fluid of an isolated frog kidney, the reaction of the secretion turns from acid to alkali, as indicated by the color of the indicator. This effect is reversible. Reasons are proposed for the assumption that the change of reaction is due to the inhibitory action of the sulfonamides upon carbonic anhydrase and that this enzyme is involved as a catalyzer in the reabsorption of bicarbonate by the kidney.

13475

Renal Physiology in Infants and Children. II. Inulin Clearances in Newborn Infant with Extrophy of Bladder.

HENRY L. BARNETT, ANNE M. PERLEY AND HELEN G. MCGINNIS.
(Introduced by Jean V. Cooke.)

From the Department of Pediatrics, Washington University School of Medicine, and the St. Louis Children's Hospital.

Interest in the renal function of newborn infants has been aroused by recent studies which indicate that the glomerular filtration rate is relatively low during the newborn period. The application of a method devised by one of us[1] to 7 apparently normal full-term infants ranging in age from 4 to 9 days suggested glomerular filtration rates between 20 and 40% of the average normal adult values. McCance and Young[2] have since published an extensive study of renal function of newborn infants, in which are included observations on inulin clearances in 3 infants, aged 6 to 13 days, with meningoceles. Urine collections were made by means of catheterization, and the determined inulin clearances were of the order of 43% of the average

adult value. These workers pointed out that the inulin clearances varied considerably with the minute urine volumes. In addition, they observed corresponding changes in urea clearances. Gordon, Harrison, and McNamara,[3] determining urea clearances in premature and full-term infants ranging in age from 7 to 70 days, also obtained values considerably below the normal adult levels. They did not find increases in the urea clearances when the flow of urine, initially 0.05 to 0.20 cc per minute or 0.4 to 1.0 cc per square meter per minute, increased by 25 to 100%.

The observations reported here were made on a 24-hour-old infant, weighing 2100 g, who was admitted to the St. Louis Children's Hospital on July 18, 1941, because of an extrophy of the bladder. The 2 ureteral orifices were visible and readily accessible. Ureteral catheters were inserted easily and a steady flow of urine was obtained. Blockage necessitating irrigation of the catheters was not encountered during the period of the studies to be described and it was felt that accurate collections were made with a minimum of manipulation. Some blood, very probably the result of trauma, appeared in the urine from the right ureter on the first day of study. On the second day of observation gross hematuria was not observed. Two groups of data were obtained: the first dealing with clearances following a single injection of inulin; the second with clearances during a sustaining infusion.

On July 18, 1941, 315 mg of inulin in the form of a 10% solution were injected intravenously and the urine was collected separately from each ureter for 3 consecutive 45-minute periods beginning 90 minutes after the injection. Blood samples were obtained at the middle of each period, and the volume of urine from each catheter was measured. During the period of the test, the patient received subcutaneously approximately 70 cc of Ringer's solution per hour. Inulin concentrations in the blood and urine were determined by a modification of the method of Corcoran and Page.[4] On July 19, after a priming dose of 1.2 cc of 10% inulin, a continuous infusion of 1.8 cc of 10% inulin in 200 cc of normal saline was given intravenously during which inulin clearances were determined for 6 consecutive 45-minute periods. The fluid intake by vein during this period was approximately 60 cc per hour. In Table

1 Barnett, H. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 654.

2 McCance, R. A., and Young, W. F., *J. Physiol.*, 1941, **99**, 265.

3 Gordon, H. H., Harrison, H. E., and McNamara, H., *Am. J. Dis. Child.*, 1941, **62**, 894.

4 Corcoran, A. C., and Page, I. H., *J. B. C.*, 1939, **127**, 608.

I are shown the data obtained for all of the periods, together with the calculated clearances. In Fig. 1 is shown the relationship between the rate of urine flow from each ureter and the inulin clearance for the corresponding kidney. The total inulin clearance for the 2 kidneys ranged from 7.30 to 21.23 cc per square meter of body surface per minute and averaged 14.17, a figure which is much lower than the average adult value of 76 cc per square meter per minute.[5] It is apparent that the inulin clearances varied considerably with the minute urine volumes, which, for the 2 kidneys, ranged from 0.078 to 0.467 cc per minute or 0.52 to 3.11 cc per square meter per minute. Although these observations are in general accord with the findings of McCance and Young,[2] the magnitude of change in clearance with change in urine flow observed by us cannot be compared directly with that observed by them since their figures for inulin clearances as related to urine flow are not corrected for surface area.

TABLE I.

Observations on Inulin Clearances for Separate Kidneys in Newborn Infant with Extrophy of the Bladder.

Period	Inulin administration	Actual urine vol. cc per min			Actual urine vol.* cc/m²/min			Inulin conc. mg%			Inulin clearances cc/m²/min		
									Urine† (diluted to 10 cc)				
		R	L	Total	R	L	Total	Plasma	R	L	R	L	Total
7-18-41													
I	Single	.049	.036	.085	.327	.240	.567	24.61	41.8	63.0	2.51	3.79	7.30
II	injection	.036	.042	.078	.240	.280	.520	18.46	44.4	104.2	3.56	8.38	11.94
III		.076	.078	.154	.507	.521	1.028	17.11	52.0	78.0	4.50	6.75	11.25
7-19													
I	Sustaining	.122	.111	.233	.814	.740	1.554	30.0	117.0	152.0	5.78	7.50	13.28
II	infusion	.178	.207	.385	1.188	1.381	2.569	25.6	137.5	153.3	7.96	8.88	16.84
III		.191	.276	.467	1.273	1.840	3.113	19.2	124.5	150.6	9.61	11.62	21.23
IV		.178	.211	.389	1.188	1.408	2.596	21.5	104.0	132.5	7.17	9.14	16.31
V		.187	.187	.374	1.248	1.248	2.496	23.7	113.5	126.5	7.10	7.91	15.01
VI		.187	.244	.431	1.248	1.629	2.877	23.3	104.0	125.0	6.62	7.94	14.56

*The value for surface area, as obtained from the nomogram[6] constructed by Hannon from the formula of DuBois and DuBois, S.A. $= Wt^{0.425} \times Ht^{0.725} \times 71.84$, was 0.15 square meters. The factor for correcting the urine volumes in cc per minute to cc per square meter per minute was, therefore, $1 \div 0.15$ or 6.67.

†Each collection period covered 45 minutes, and each urine specimen was originally made up to 10 cc before the determination of the inulin concentration was undertaken. (Urines III L and VI L of 7-19-41 were diluted to 20 cc and the inulin concentrations corrected to 10 cc.) The diluted urine volume corrected for surface area and for time was, therefore, $10 \div 45 \times 6.67$ or 1.481 cc per square meter per minute. This figure was used for V in the formula, UV/B, for the calculation of each of the inulin clearances.

[5] Goldring, W., Chassis, H., Ranges, H. A., and Smith, H. W., *J. Clin. Invest.*, 1940, **19**, 739.

[6] DuBois, E. F., *Basal Metabolism in Health and Disease*, p. 124, Lea & Febiger, Philadelphia, 1927.

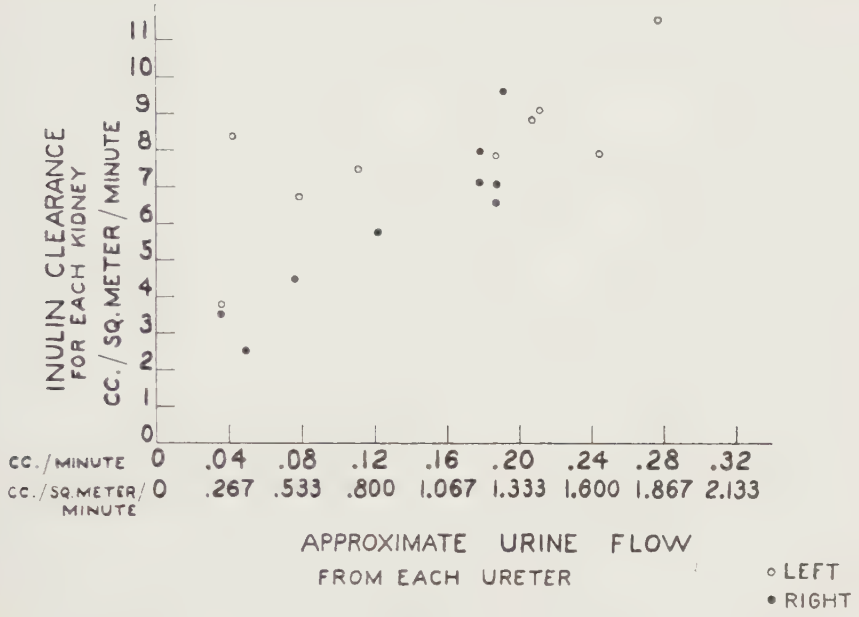

FIG. 1.

Relation between rate of urine flow from each ureter and the inulin clearance for the corresponding kidney.

These observations on a 24-hour-old infant with extrophy of the bladder* offer further evidence that the inulin clearance in newborn infants is considerably lower than in adults. In general, throughout the range of urine flow observed, the inulin clearances varied directly with the minute urine volumes.

* An autopsy on July 22, 1941, revealed that the extrophy of the bladder was accompanied by congenital defects of other organs, but the kidneys and urinary tract showed no other anomalies.

13476 P

Characterization of Milk Influence in Spontaneous Mammary Carcinoma.

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Previous studies[1-6] indicate the presence, in at least certain tissues of high cancer strain mice, of a substance or substances whose presence is essential to spontaneous mammary carcinoma. In these studies lactating mammary tissues inclusive of their contained milk have been brought into suspension and partial solution by microhomogenization and the resulting fluid separated into several fractions by the use of an ultracentrifuge.

Procedure. Fostered female C_3H mice 1 month old were employed as test animals. Lactating breast tissues were obtained immediately after death by etherization, from high cancer C_3H and A strain mice. The material was immediately homogenized with addition of between one and two times its volume of sterile distilled water. The resulting fluid was spun in the centrifuge at 15,000 rpm, giving a maximum accelerational force of 15,000 x g, for 30 minutes. Three layers resulted, a fatty top layer, designated as the fat fraction, a middle aqueous layer, the first supernatant fluid, and a bulky precipitate, the first sediment. The fat fraction was removed and employed as noted below. The aqueous layer was removed by suction without disturbing the sediment. The first supernatant fluid was then spun at 40,000 rpm, corresponding to 110,000 x g, for a period of 60 minutes. The final supernatant fluid was removed without disturbing the second sediment.

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† Aided by grants from the Carnegie Corporation and the Cancer Institute Committee of the University.

‡ Aided by grants from the National Cancer Institute and the Jane Coffin Childs Memorial Fund for Medical Research.

1 Bittner, J. J., *Science*, 1936, **84**, 162.

2 Bittner, J. J., *Public Health Reports*, 1939, **54**, 1827.

3 Bittner, J. J. PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 805.

4 Bittner, J. J. *Cancer Research*, 1941, **1**, 290.

5 Bittner, J. J., *Science*, 1941, **93**, 527.

6 Andervont, H. B., and McEleney, W. J., *J. Nat. Cancer Inst.*, 1941, **2**, 13.

The materials used for feeding or injection were (1) homogenized tissue, (2) fat fraction, (3) first sediment, (4) second sediment, and (5) final supernatant fluid. The amount of material of each fraction which was injected or fed was usually that which was obtained in that fraction from 1 g of original tissue. The materials were administered by one of two means: (1) by subcutaneous injection, or (2) by stomach tube. All mice were maintained on Purina Fox Chow, *ad libitum*. Lettuce was supplied at intervals. Each animal was allowed to deliver 5 litters and to carry them to weaning in most instances.

Results. Eighty-four mice were given one or another of the 5 materials listed above, either by subcutaneous injection or by stomach tube, and survived the administration. There was a heavy immediate mortality in animals injected with the homogenized tissue and the first sediment. The incidence of verified mammary carcinoma in these animals at age 12 months is given in Table I. It will be noted that the highest incidence occurs in the group given homogenized tissue, and that the second sediment animals showed a somewhat smaller, but still very large incidence. The first sediment, fat fraction, and supernatant fluid, produced progressively smaller cancer incidence rates.

Centrifugation at 15,000 rpm removed from the aqueous phase nearly all of the large particulate matter. The disperse phase in the first supernatant fluid consists essentially of colloids and crystalloids in solution. Centrifugation at 40,000 rpm for one hour sediments a large fraction of the higher molecular (particle) size dispersed material. Exactly what fraction, in the case of the materials here employed, has not been determined. The second sediment consists mainly of the larger particle size colloidal material, probably largely protein. If a virus protein were present in the material it is very likely that it would be sedimented in this fraction. The low cancer incidence in 22 mice given the fat fraction is believed to indicate that a little of the active material is occluded with the low density fatty material, rather than that the fat itself possesses carcinogenic activity. The lower incidence of cancer in animals given

TABLE I.

Material	No. of mice	No. with tumors	% with tumors	Avg tumor age, mo.
Homogenized tissue	14	10	71	9
Fat fraction	22	3	14	10
First sediment	17	7	41	9
Second "	20	11	55	10
Final supernatant	11	0	0	—

first sediment, than in those given second sediment or homogenized tissue may have one of several interpretations. It is possible that less of the active material is actually sedimented, because of ready solubility. On the other hand, it is equally possible that the large amount of inert material associated with active material exerts either directly or indirectly an inactivating effect.

The observations reported appear to show: (1) That the active carcinogenic agent or agents in the lactating breast from high cancer mice are present in a fraction obtained by ultracentrifugation of homogenized tissue, which fraction is virtually free of matter above colloidal dimensions, and contains a large share of the soluble material of high molecular weight. (2) That the active material in the above-mentioned fraction is either greatly concentrated by the procedures employed or that interfering substances are removed. (3) That the agent or agents in question appear in traces if at all in the fat fraction and in the final supernatant fluid. The exact nature of the active agent is not certain from these studies, but it becomes very probable that the agent is a colloid of high molecular weight and may be a virus. Further studies on a larger series of animals are in progress.

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Transfusion of Bovine Serum Albumin into Human Beings.

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In a previous communication[1] it was reported that the intravenous administration of bovine serum albumin was effective in raising and maintaining the blood pressure of dogs subjected to severe hemorrhage. The present report is concerned with the effects of transfusion of bovine serum albumin into human beings.

Materials and Methods. The serum was obtained from bovine blood and centrifuged. The supernatant serum was removed, pooled and passed through a Seitz filter. The pooled serum was treated with an equal volume of a saturated solution of ammonium sulfate (54%). This mixture was now filtered through a Buchner filter which removed the precipitated globulin. The filtrate was fully sat-

[1] Davis, H. A., and Eaton, A. G., PROC. SOC. EXP. BIOL. AND MED., in press.

urated with ammonium sulfate and refiltered through a Buchner filter. The precipitated albumin was then placed in cellophane cylinders and dialyzed against distilled water to remove completely the ammonium sulfate. In the present series of experiments the albumin content of the serum from analysis was 5 g %. The serum albumin was passed through a Seitz filter and finally through a Berkefeld filter. Tests for sterility were run on each pooled lot of bovine serum albumin, using as media, infusion broth, brain broth, plain agar and blood agar plates. The intraperitoneal injection of the serum albumin into white mice in relatively large amounts (1 cc per 15 g of body weight) revealed a complete absence of toxic effects.

The human subjects varied in age from 15 to 65 years. Intradermal injections of the serum albumin (1 cc) were made in each subject to determine the presence of sensitiveness to the serum. Blood pressure readings were made before, during and after the injections. The serum albumin was administered by vein in amounts of 50 cc to 300 cc at a rate of 5 cc per minute. Preliminary cross-matching of the serum albumin with the blood of the prospective recipient was not carried out.

Results. The effects of the transfusion of bovine serum albumin in 13 human subjects are illustrated in Table I. The blood pressure remained unchanged or rose slightly during the transfusion. No evidences of a foreign protein reaction such as dyspnoea, urticaria or fall in blood pressure were observed. In none of the patients was albuminuria noted. Rigors and elevations of the temperature did not occur. Out of 16 patients who were given

TABLE I.
Effects of Transfusion of Bovine Serum Albumin in Human Beings.
Intradermal test 1 cc, all negative; urinalysis, no albuminuria; result, negative.

Patient	Age, yrs	Amt bovine serum albumin, cc	Blood pressure		
			Before inj., mm Hg	During inj., mm Hg	After inj., mm Hg
R.R.	23	50	134/93	130/98	131/94
C.J.	15	50	130/68	138/78	130/60
H.D.	43	50	118/95	136/84	128/80
C.J.	57	50	158/100	158/100	158/100
W.H.	63	50	138/74	150/78	142/74
E.M.	55	100	100/68	98/60	104/74
H.S.	39	100	140/110	144/106	140/110
C.S.	57	100	106/88	114/74	106/74
L.W.	37	100	142/88	144/88	144/88
H.P.	31	200	106/56	110/62	106/58
E.G.	26	200	124/70	128/80	132/74
E.J.	65	200	98/58	98/56	106/60
R.S.	27	300	114/80	120/86	114/64

the intradermal test injections (1 cc) only 2 showed a doubtful positive local reaction (Grade 1).

Comment. The absence of toxic effects following the transfusion of bovine serum albumin into human beings and into mice tends to substantiate our previous statement[1] that it is the globulins of bovine serum which are responsible for the toxicity of the whole serum. The results of the transfusion of bovine serum in human beings are promising from the viewpoint of its use as a substitute for blood. The absence of albuminuria following these transfusions possibly indicates that bovine serum albumin is utilized by the human being.

Summary. The intravenous administration of bovine serum albumin to 13 human beings has given encouraging results, inasmuch as no reactions were noted. The blood pressure was maintained at, or rose above, the initial level. Vasodepression was not observed. The significance of these results in relation to the use of bovine serum albumin as a blood substitute in human beings is discussed.

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A Study of the Effect of Desoxycorticosterone Acetate on Capillary Permeability.*

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The adrenal cortical hormones may increase the circulating plasma volume in adrenal insufficiency (1) by the control of renal function and (2) by the transfer of water and electrolytes to the blood stream from the tissues.[1] Whether these mechanisms operate by an alteration of capillary permeability is not known. The purpose of this communication is to report data bearing on this point.

The diffusion of dyes from the blood stream was utilized as a means of observing the capacity of desoxycorticosterone to influence the flow of fluids across the capillary membrane. Normal white rabbits were given one intravenous dose of one of 3 different dyes: trypan blue, brom phenol blue or patent blue V.† For each rabbit

* Aided in part by a grant from the Proctor Fund, Harvard Medical School.

1 Kendall, E. C., *Proc. Staff Meetings, Mayo Clinic*, 1940, **15**, 297.

injected with a definite dose of a given dye, a corresponding white rabbit, which had received desoxycorticosterone acetate‡ parenterally several hours in advance, was observed simultaneously. The rate of appearance of dye in the ears, mucous membranes, conjunctivae and skin of the abdominal wall was noted and compared with the controls.

Obvious delay in the appearance of trypan blue in these areas in animals under the influence of desoxycorticosterone acetate was noted. The difference was marked not only in the time of appearance but in the intensity of staining. Although there is no way of quantitating these differences, agreement was uniform as to the reality of the differences to 6 different observers, who rendered judgment without knowing which of the pair had received the hormone.

In the case of brom phenol blue the difference in time and intensity were in the same direction, though less marked.

No such striking difference was noted with patent blue V (and in a few instances with eosin and fluorescin). But a striking difference was noted in the time of disappearance of patent blue V, in that the rabbit which received hormone retained its greenish blue color for a perceptibly longer period in 3 out of 4 experiments (Table I).

The difference in staining of tissues under the influence of desoxycorticosterone acetate was not due to chemical alteration of the dye by the hormone because there was no difference in the concentration of dye (as measured by the photoelectric colorimeter) in samples of blood taken at intervals from the hormone-treated and untreated rabbits or *in vitro*, using horse serum or phosphate-buffered physiological saline solution (pH 7.4) with appropriate concentrations of desoxycorticosterone acetate and dye.

The specificity of desoxycorticosterone acetate in these reactions was shown by substituting testosterone propionate or progesterone, which are ketosteroids closely related to desoxycorticosterone in chemical structure, in three rabbits. These showed the same appearance time and intensity of staining with trypan blue as control rabbits. It is therefore clear that desoxycorticosterone acetate possesses an effect on the diffusion of dyes which is apparently specifically related

† These dyes, in the order named, possess increasing degrees of diffusibility: trypan blue a vital dye, being the slowest to appear in the tissues, while patent blue V, like eosin, is so rapidly diffusible that the normal animal usually becomes intensely blue before the injection of the dye solution is completed. The disappearance of these dyes from the tissues is of a corresponding order: patent blue V is completely excreted as a rule within an hour, while trypan blue remains fixed in the tissues for many days.

‡ Kindly furnished by Schering Corporation under the trade name "Cortate" and by the Ciba Co. under the trade name "Percorten."

TABLE I.

The Effect of Desoxycorticosterone Acetate on the Diffusion of Intravenously Injected Dyes in Rabbits.

Exp. No.	Dye (in distilled water)	Amt of dose in mg	Dye		Time of appearance of dye after injection		Time of disappearance of dye after injection	
			Amt, cc	Conc., %	Control	Hormone	Control	Hormone
1	Trypan Blue	1	2.5	1	immediate	40 min	10-14 days	10-14 days
2	" "	1	2	1	"	45 "	10-14 "	10-14 "
3	" "	1	3	1	"	a. 45 " b. 50 "	10-14 "	10-14 "
4	" "	5	2	1	8 min	a. 45 " b. 2½ "	— died 10 hr after inject.	10-14 "
5	" "	5	1	0.5	30 "	3 hr	10 days	10 "
6	Bromphenol Blue	2	3	1	30 "	60 min	5 "	5 "
7	" "	2	3	1	3 "	a. 12 " b. 12 "	5 "	5 "
8	Patent Blue V.	1	2	1	immediate	a. immediate b. "	3 hr	a. 2½ hr
9	" " "	5	1	1	"	"	½ "	1 "
10	" " "	10	1	0.5	"	5 min	20 min	1 "
11	" " "	10	1	0.3	"	3 "	40 "	1½ "
12	Eosin	1	2	5	"	a. 16 " b. 15 "	2-3 days	2-3 days
13	Fluorescin	2	1.8	10	"	immediate	1 hr	1 hr
14	"	5	1.6	10	"	"	1 "	1 "

to the $COCH_2OH$ radical attached to the phenanthrene ring.

In vitro experiments on the diffusion of these dyes showed no uniform effect from the presence of the hormone, but certain clear-cut differences appeared when the intact frog or the surviving frog's skin were used as diffusion membranes.

It is clear from the fact that trypan blue is not excreted by the kidneys that these dye phenomena cannot be related to renal function.

That this effect on the diffusion of dyes is not related to an alteration in capillary permeability is suggested by the following observations:

I. In 2 normal subjects the rate of disappearance of intravenously injected NaCNS from the blood stream was not altered by the intramuscular injection of 25 mg of desoxycorticosterone acetate 3 hours previously.

II. In 2 normal subjects the time of disappearance of an intracutaneous wheal produced by injecting physiological saline solution was not altered by the intramuscular injection of 25 mg of desoxycorticosterone acetate.

III. In 3 normal subjects the rate of appearance and the size of the wheal and flare at the site of injection of 1/10 cc of 1/1000 solution of histamine hydrochloride was the same before and 3 hours after the intramuscular injection of 25 mg of desoxycorticosterone acetate.

IV. In a case of nephrosis the average 24-hour loss of albumin in the urine was substantially the same for 10 days preceding and for 6 days during which a daily intramuscular dose of 25 mg of desoxycorticosterone acetate was administered.

V. In dogs the rate of absorption of water, isotonic sodium chloride or glucose from loops of ileum or colon was not altered appreciably by the intravenous injection of 5 mg of desoxycorticosterone.

These observations do not support the concept that desoxycorticosterone acetate has an effect on capillary permeability to water and electrolytes.

Desoxycorticosterone acetate produced no change in the concentration of plasma proteins in the following 3 subjects: (a) a normal person, (b) a patient with nephrosis, and (c) a patient with probable cirrhosis of the liver.

The negative effect of this hormone on the function of the normal capillary indicates that its positive effect, as in adrenal insufficiency and on the diffusion of dyes, is exerted elsewhere. Menkin[2] reported

[2] Menkin, V., *Am. J. Physiol.*, 1940, **129**, 691.

that the permeability of capillaries damaged by the local injection of irritants was altered, as indicated by the local diffusion of intravenously injected trypan blue, by the local injection of desoxycorticosterone acetate. We were unable to confirm these findings when this hormone was injected intravenously.

If the primary pathology in traumatic shock is an altered permeability of the capillaries, the foregoing observations are consistent with evidence recently presented by us that desoxycorticosterone acetate exerted no beneficial effect in shock following hemorrhage.[3]

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Fixation of Iodine by Thyroids of Rats Given Diets Deficient in Iodine.*

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Iodine fixation in the thyroids of rats given a goitrogenic diet was examined with the help of radio-iodine. One group of animals received Remington's diet No. 342,[1] modified after Steenbock and Black's diet No. 2965[2-5] by adding liver extract to improve growth, and by reducing the amount of calcium, so that the results could be ascribed to the low iodine content of the diet and not to the goitrogenic effect of calcium.[1] The amount of iodine estimated by the method of Matthews, *et al.*,[6] after destruction according to Kolnitz and Remington,[7] was found to be 19 μg per kilo. The animals ate

[3] Fine, J., Fischmann, J., and Frank, Howard A., *Surgery*, in press.

* This research was supported by a grant-in-aid from the Rockefeller Foundation administered by Dr. Stafford Warren. It was completed with the help of a Hoffman-LaRoche Fund, administered by Dr. Hans Selye. The radioactive iodine was kindly furnished by Dr. R. T. Evans, from the cyclotron of the Massachusetts Institute of Technology.

[1] Remington, R. E., *J. Nutr.*, 1937, **13**, 223; Remington, R. E., and Levine, H., *J. Nutr.*, 1936, **11**, 343.

[2] Steenbock, H., and Black, A., *J. Biol. Chem.*, 1925, **64**, 263.

[3] Krauss, W. E., and Monroe, C. F., *J. Biol. Chem.*, 1930, **89**, 581.

[4] Thompson, J., *J. Nutr.*, 1932, **5**, 359.

[5] Levine, H., Remington, R. E., and von Kolnitz, H., *J. Nutr.*, 1933, **6**, 325.

[6] Matthews, N. L., Curtis, G. M., and Brode, W. R., *Ind. Eng. Chem.*, 1938, **10**, 612.

[7] Kolnitz, H. v., and Remington, R. E., *Ind. Eng. Chem.*, 1933, **5**, 38.

an amount of food containing approximately 0.2 μg of iodine daily, which is well below the minimum daily iodine requirement of the rat.[1]

Another group of rats was given Sharpless' diet No. 153,[8] in which there was soybean, an active goitrogenic agent.[9, 10, 11] The amount of iodine in the diet was found to be 1 μg per kilo, so that in this case the animals, in addition to the action of a goitrogenic agent, had a diet very deficient in iodine.

Ten males and 12 females were put on each diet and divided into equal groups of control and experimental animals. The controls received distilled water containing 2 μg of iodine per cc, while the experimental animals received double-distilled water. The amount of iodine consumed by the controls varied, but was in the vicinity of 20 μg of iodine daily.

Both control and experimental animals gained weight on the Remington diet, but growth was poor and diarrhea frequent with the Sharpless regime. After 70 days (Remington's diet) or 54 days (Sharpless' diet), all the animals received an intraperitoneal injection of 15 μg of radio-iodine in the form of sodium iodide. One animal of each group was sacrificed one hour later. Since only about 2% of the injected radio-iodine was then fixed in the thyroids of the controls, and 4% in those of the experimental animals, it was decided to sacrifice the other animals at a later time. This was done either 8 or 24 hours after the injection of radio-iodine. The results, being similar at these two periods, were reported together (Table I).

At autopsy the hypophysis and a piece of the thyroid were removed for histological examination. Each thyroid was then destroyed in a mixture of chromic and sulphuric acids, and its radio activity estimated on a Geiger counter. Then the products of thyroid destruction from the animals of one group were pooled and the total iodine content estimated. In the table, the total iodine values are given after subtraction of the radio-iodine.

Results. (Table I.) In confirmation of previous work,[1, 3–5, 9] hyperplasia of the thyroid was shown by an increase in the weight of the gland, by thickening of the epithelium of the follicles with a decrease in the amount and density of the colloid, and by a decrease in the total iodine content of the organ. The amount of radio-iodine fixed by the thyroid gland was greater in the experimental than in the

8 Sharpless, G. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 166.

9 Sharpless, G. R., Pearsons, J., and Prato, G. S., *J. Nutr.*, 1939, **17**, 545.

10 McCarrison, R., *Ind. J. Med. Res.*, 1933, **21**, 179.

11 Wilgus, H. S., Jr., Gassner, F. X., Patton, A. R., and Gustavson, R. G., *J. Nutr.*, 1941, **22**, 43.

TABLE I
Fixation of Radio-iodine by Thyroids of Rats on Iodine Deficient Diets.

	Avg body wt, g		Avg wt thyroid (mg)	Conc. of iodine in thyroid (mg per 100 g)	Avg conc. of radio-iodine in thyroid (mg per 100 g)	% of injected radio-iodine in thyroid (avg)
	Beginning	Autopsy				
I. Remington's Diet						
♂ Controls	119	234	12.8 (10.5-15.5)	100	7.0 (5.7-8.2)	4.8 (3.9-5.8)
Exp.	121	224	27.0 (23-32)	14	11.4 (6.1-19)	20.5 (7.2-37.2)
♀ Controls	91	177	14.5 (11.5-18.5)	—	6.2 (3.8-7.8)	5.9 (3.4-7.7)
Exp.	91	170	16.2 (13-19)	—	14.2 (8.5-18.8)	18.2 (11.3-25)
II. Sharpless' Diet						
♂ Controls	169	163	12.5 (10-15)	138	3.9 (3.7-4.3)	4.9 (4.6-5.2)
Exp.	199	202	18.0 (15-21.5)	75	32.6 (24.3-39.6)	43.7 (30-49.1)
♀ Controls	97	100	14.0 (10-19)	64	6.5 (4.4-9.6)	7.0 (5-8.1)
Exp.	141	142	20.3 (16.5-24.5)	34	11.5 (4.1-20.2)	20.1 (7.8-33.4)

The values in parentheses indicate the extreme variations.

control animals. Therefore, the transformations of the thyroid due to iodine deficiency result in a better use of the available iodine and thus are beneficial to the animals. The results were similar in the case of the straight iodine deficiency (Remington's diet) and in the case of the soybean diet, suggesting that the thyroid hyperplasia of iodine deficiency was not modified when a goitrogenic agent was present in the diet. It has also been shown that the action of goitrogenic factors in rabbits, such as obtained by feeding cabbage or injecting cyanides, also produces hyperplastic thyroids, fixing increased amounts of iodine.[12]

It must be noted that the thyroids made hyperplastic by dietary means behave as if they were stimulated by thyrotropic hormone, since the action of this hormone on the thyroid is to produce an increased ability to fix iodine,[13-15] and histological signs of hyperplasia.[16] Indeed, a hypersecretion of thyrotropic hormone by the

[12] Hertz, S., Roberts, A., Means, J. H., and Evans, R. D., *Am. J. Physiol.*, 1939, **128**, 565.

[13] Leblond, C. P., and Süe, P., *C. R. Soc. Biol.*, 1940, **133**, 543; Leblond, C. P., and Süe, P., *Am. J. Physiol.*, 1941, **134**, 549.

[14] Hamilton, J. G., and Soley, M. H., *Proc. Nat. Acad. Sc. U. S.*, 1940, **26**, 483; Hertz, S., and Roberts, A., *Endocrinol.*, 1941, **29**, 82.

[15] Morton, M. E., Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, 1941, **140**, 603.

[16] Loeser, A., *Arch. Exp. Path. Pharm.*, 1936, **184**, 23.

hypophysis of iodine deficient animals was suggested by histological examination of their hypophysis. These glands showed a decreased percentage of acidophils with an increased number of basophils, as is the case with thyroidectomized animals.[17, 18] Since this hypophyseal picture in thyroidectomized animals is indicative of excessive release of thyrotropic hormone,[16, 19, 20] it is likely that it bears the same significance in animals lacking iodine. Goitrous thyroids would thus result from hypophyseal modifications. However, the possibility of a direct action of iodine on the thyroid is not excluded.[21]

Conclusion. The hyperplastic thyroids of rats given an iodine-deficient diet acquire an increased ability to fix iodine. The thyroid and hypophyseal changes in these goitrous animals indicate an excessive release of thyrotropic factor from their hypophysis.

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Use of Hamster (*Cricetus auratus*) for Detection of Influenza Virus in Throat Washings.*

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One of us has reported[1] that the hamster *(Cricetus auratus)* gave a specific immune response following intranasal inoculation of throat-washings taken from persons acutely ill with influenza A. These observations have now been extended, not only with regard to the immune response of the hamster but also as to its use in adapting influenza A virus to mice.

Experimental Material. The throat-washings used in these experiments were collected from persons presumably ill from influenza during the epidemic which occurred in the Argentine in July, 1940. The throat-washings were taken with 20 cc of an equal mixture of

17 Marine, D., Rosen, S. H., and Sparke, C., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 803.

18 Sharpless, G. R., and Hopson, E. M., *Endocrinol.*, 1940, **27**, 129.

19 Loeser, A., *Arch. Exp. Path. u. Pharm.*, 1934, **176**, 697.

20 Starr, P., Rawson, R. W., Smalley, R. E., Doty, E., and Patton, H., *West. J. Surg.*, 1939, **47**, 65.

21 Chapman, A., *Endocrinol.*, 1941, **29**, 680.

* These studies were supported in part by a grant from the International Health Division of the Rockefeller Foundation.

1 Taylor, R. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 541.

0.85% NaCl and buffered broth (pH 7.4). They had been preserved for a period of 6 to 8 months at a temperature of approximately –75°C in a thermos or cold cabinet[2] containing CO_2 ice.

For the purpose of serological diagnosis, acute and convalescent blood samples were taken from each person from whom a throat-washing was secured.

Method. Inoculation. The hamsters were inoculated intranasally with 0.4 cc of throat-washings while under light ether-anesthesia. As it had been shown that the intranasal instillation of a bland fluid would increase the quantity of virus in the lungs of mice which had previously received a sublethal dose of influenza A virus,[3] the hamsters were again placed under light ether-anesthesia 2 to 3 days after the inoculation of the throat-washings, and 0.4 cc of 0.85% NaCl solution was instilled into the nose.

The ferrets used for comparison were similarly inoculated with 1 cc of the throat-washings. No second instillation was given to these animals.

From animals used for testing the immune response, a blood sample was taken by cardiac puncture preceding and 2 weeks following inoculation.

The *neutralization-test* was employed for determining the immune response by inoculating mice with varying dilutions of the serum mixed with a standard quantity of virus. It was found advisable to use a relatively small dose of virus, not exceeding 100 MLD to detect consistently the increase of neutralizing antibodies in the sera of the hamsters.

Mouse-passage. For the adaptation of the virus to mice, the hamsters were sacrificed by chloroform-anesthesia on the fourth day following inoculation of the throat-washings, and the turbinates and lungs were removed with aseptic precautions. The turbinates were ground in a porcelain mortar with a small amount of alundum and suspended in 0.75 cc of buffered broth (pH 7.5). The lungs were likewise emulsified and suspended in 4 volumes by weight of broth. Following centrifugation, equal quantities of the supernate of the turbinate- and lung-suspensions were mixed and 0.05 cc was inoculated intranasally into each of 4 mice while they were under ether-anesthesia. On the second or third day 0.05 cc of 0.85 NaCl solution was similarly administered intranasally. On the fourth day the mice were sacrificed. Their lungs were emulsified as above described, and with this suspension 4 additional mice were inoculated,

[2] Horsfall, F. L., Jr., *J. Bact.*, 1940, **40**, 559.

[3] Taylor, R. M., *J. Exp. Med.*, 1941, **73**, 43.

and the passages continued until 5 passages had been completed. If pulmonary lesions simulating viral infection were observed, further passages were made until the virus was sufficiently virulent for titration against a known immune serum which constituted the final criterion for identification of the virus.

Results. Twelve throat-washings were administered to ferrets and hamsters to determine the immune response in these animals. Ten were from persons who according to serological diagnosis had influenza A. Nine of these 10 produced a positive reaction to influenza A virus in the ferret and hamster, while one was negative to both species of animals. One throat-washing was from a person with an influenza B infection and elicited an immune reaction to the B virus in the ferret as well as the hamster. The remaining throat-washing was taken from a person who, following the attack, showed a rise in neutralizing antibodies to influenza A and B virus, but produced a positive response to influenza virus only in both the ferret and hamster. Thus there was complete accord in the immune reaction of the ferrets and hamsters.

Attempt was made to adapt the virus to mice after one hamster-passage with 5 of the throat-washings which had produced an immune reaction to the A virus. In each instance following 3 to 4 mouse-passages, pulmonary lesions began to appear, and on further passages the virulence increased and the mice began to die with typical pulmonary consolidation. It was subsequently shown that the agent producing these lesions was filtrable, and was neutralized by influenza A virus immune serum.

An effort to transfer from a hamster to mice the virus identified as influenza B virus by the immune response, was not successful. The failure may be attributed to the presence of a "wild virus," which seemingly had its origin in the hamster. This virus became rapidly fatal to mice, and would probably have masked an infection of influenza B virus, had the latter existed.

Discussion and Summary. The comparative inoculations which have been made in hamsters and ferrets indicate that the hamster may be substituted for the ferret in identifying influenza A virus in throat-washings, either through the production of specific neutralizing antibodies to this virus, or as the first step in adapting the virus to mice. The one throat-washing at our disposal which contained type B influenza virus, likewise produced an immune response in the hamster; but owing to a contaminating "wild virus" success was not achieved in adapting the type B influenza virus to mice after a passage in the hamster.

It should be emphasized, however, that the titer of the hamster serum following infection is usually not so high as that of the ferret and it is therefore advisable to use not more than 100 MLD of virus in performing the neutralization-test.

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A Selective Medium for Isolation of *Hemophilus influenzae*.

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The isolation of Gram negative pathogenic microörganisms from the nasopharynx is often difficult because of overgrowth by Gram positive cocci. This difficulty is increased when the organism requires an enriched medium which favors the growth of normal flora. Fleming[1] inoculated plates with penicillin and noted that Gram positive cocci could be inhibited and *H. influenzae* obtained in relatively pure culture.

The determination of the incidence of *H. influenzae* in the nasopharynx, because of its possible relation to the severity of influenza epidemics, is of considerable importance. A more convenient method of isolation than those available at present would be of obvious value. Accordingly we investigated the effect on this organism of the bactericidal agent, tyrothricin, isolated by Dubos.[2]

The tyrothricin was prepared according to the method of Dubos.[2, 3, 4] Following saline precipitation, it was kept in the form of a stock alcoholic solution (8 mg/100 cc). The tyrothricin was diluted in 5% glucose in distilled water. It was incorporated into the medium by placing 1.5 cc of the dilution to be studied in a Petri plate and adding 15 cc of Fildes' peptic blood digest agar at 45°C. The plates were allowed to dry at room temperature and 0.1 cc of inoculum spread over the surfaces. Chocolate agar plates were similarly made containing tyrothricin. A heavy inoculum of *Staphylococcus aureus*, mixed with varying dilutions of *H. influenzae* was used.

1 Fleming, A., *Brit. J. Exp. Path.*, 1929, **10**, 226.

2 Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1.

3 Dubos, R. J., and Hotchkiss, R., *J. Biol. Chem.*, 1940, **136**, 803.

4 Dubos, R. J., and Hotchkiss, R. D., *J. Exp. Med.*, 1941, **73**, 629.

It is clear from the data included in Table I that *H. influenzae* is resistant to the action of tyrothricin, and that by incorporation of this substance in the medium a selective killing of this strain of staphylococcus was obtained. It will be noted that in the higher dilutions of tyrothricin, due to the obscuring effect of the staphylococcus only a few colonies of *H. influenzae* were evident. The number of colonies of influenza bacilli was not reduced even in a 1:100 dilution of the stock alcoholic tyrothricin solution, although at this concentration the colonies were somewhat smaller and showed less iridescence. The bacilli from the medium containing tyrothricin exhibited typical morphology, reacted with capsular swelling in type-specific rabbit serum, and were still viable on transfer after 6 days.

Attempts to use the tyrothricin in the starch casein hydrolysate medium of Mueller and Hinton[5] for selective isolation of meningococci and gonococci were unsuccessful. Tyrothricin inhibited the growth of these Gram negative cocci and staphylococci in the same dilutions. Further purification and the use of recrystallized gramicidin also failed to give differential inhibition of the staphylococci. Dubos[6] has noted that gramicidin kills the meningococcus and the gonococcus. This bactericidal action is apparently an exception to

TABLE I

Effect of Tyrothricin on Mixed Cultures of Staphylococcus and *H. influenzæ*

Inoculum*	Final dilutions of tyrothricin in plates of peptic digest agar				
	1:110	1:1100	1:11,000	1:110,000	1:1,100,000
Staphylococcus 10^{-2}	0	0	+	+++	++++
H. influenzæ 10^{-6}	72 col.†	Colonies not well separated	43 col.	76 col.	75 col.
Staph., 10^{-2} } Flu., 10^{-6} }		78 col. *H. influenzæ*. No staph.	Moderate growth of staph. Scattered col. *H. influenzæ*	Heavy staph. No *H. influenzæ* visible	Heavy staph. No *H. influenzæ* visible
Staph., 10^{-2} } Flu., 10^{-4} }	*H. influenzæ* only†	*H. influenzæ* only	Mixed *H. influenzæ* and staph.	Heavy staph. Occasional col. *H. influenzæ*	Heavy staph. Occasional col. *H. influenzæ*

*By plate count without tyrothricin 0.1 cc of 10^{-2} dilution of staphylococcus culture contained 196,000 organisms.

0.1 cc of 10^{-6} dilution of *H. influenzæ* culture contained 68 organisms.

†At this concentration of tyrothricin, the colonies of mucoid *H. influenzæ* were reduced in size, and showed markedly less iridescence.

5 Mueller, J. H., and Hinton, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 330.

6 Dubos, R. J., *The Harvey Lectures*, 1939-1940, p. 223.

the restriction of the activity of gramicidin to Gram positive organisms.

The media containing tyrothricin were then tested for the cultivation of material obtained from apparently healthy subjects. The region of the pharynx was swabbed with a sterile cotton-tipped applicator which was then placed in about 1 cc infusion broth. After incubation for 1 hour a drop of the broth was placed on each of four media: Fildes' peptic digest agar, Fildes' peptic digest agar containing tyrothricin (10^{-3}), chocolate agar and chocolate agar containing tyrothricin (10^{-3}). The difference between the media containing tyrothricin and the ordinary media was strikingly apparent. On chocolate media the change in color about streptococci and staphylococci was intense and few individual colonies were apparent, while the media containing the tyrothricin revealed no discoloration and isolated colonies were easily discerned. The peptic digest medium containing tyrothricin was also more satisfactory than its control. Gram positive organisms were conspicuously absent.

Isolation of *H. influenzae* was greatly facilitated and a somewhat larger number of isolations were made on the media containing tyrothricin than on the others. Although the ease of isolation cannot be illustrated in a table, the results of the number of *H. influenzae* isolated in each case are of interest (Table II).

No attempt was made to pick colonies that might resemble *H. hemolyticus* although in a number of cases this organism was isolated.

Conclusion. Through the use of a medium containing tyrothricin in a concentration inhibitory to Gram positive cocci, isolation of *H. influenzae* from the nasopharynx may be facilitated. This medium is being tested in epidemiological surveys now in progress.

TABLE II.
Isolation of *H. influenzæ* on Media with and without Tyrothricin.

	No. cultures examined	*H. influenzæ* isolated
Tyrothricin in Fildes' medium	130	23
Fildes' medium alone	86	10
Chocolate and tyrothricin	86	6
Chocolate alone	86	0

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